IRON METABOLISM IN LAYING HENS

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by

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IRON METABOLISM

INTRODUCTION

Although the therapeutic value of iron has been recognised since ancient times, the conception that its metabolism differs from that of other nutritional elements in its divergence from the usual pattern of absorption, utilisation and excretion, has only emerged in the last quarter of a century with the introduction of precise chemical and radioactive analytical procedures.

Intake of iron is governed not only by availability but also by the limited power of the body to excrete the element, absorption consequently being limited by functional requirements.

Within the body priority in the demand for iron is accorded to the respiratory enzymes essential to cell metabolism and to myoglobin, the oxygen storage compound of muscle, so a deficiency of these is rarely observed.

The remainder of body iron is utilised for the circulatory haemoglobin whose chief function is to transport oxygen to the tissues for cellular oxidative purposes.

The plasma is concerned with the transport of iron throughout the body, so that abnormalities of iron metabolism are readily reflected by changes in plasma iron. Since plasma iron is bound to a specific protein, alteration in the concentration of the latter by increasing or decreasing the iron combining capacity of the plasma, may also influence plasma iron concentration. Iron which is not immediately

required for metabolism enters the storage depots in the liver, spleen and bone marrow and is stored there either as haemosiderin or ferritin.

Absorption

Absorption of iron is believed to occur in the stomach, duodenum, and upper jejunum (Granick 1949). McCance and Widdowson (1938) give the daily dietary requirements as 5-8 mg. for the human male and twice this amount for adult females. Moore (1955) incorporated radioactive iron into various foods, and by this technique was able to show that on an average only some 10 per cent of iron in foodstuffs is absorbed, and that as the average daily diet contains 12-15 mg. of iron, the adult male absorbing some 1.5 mg. of the element maintains an easy iron balance, but the female with a menstrual loss of 35-70 cc. of blood, equivalent to 14-28 mg. of iron, is in an uneasy state of iron balance until the menopause.

In the nineteen twenties, controversy arose over the nutritional value and availability of ferric and ferrous salts, e.g. Mitchell and Schmidt (1926) who first suggested the terms soluble and insoluble iron in preference to organic - from biological material - and inorganic iron. The school of Elvehjem and Hart published some ten papers on iron in nutrition and anaemia in the rat, claiming that chemical inorganic iron was ineffective while iron from organic material was of value and suggested that any

successes in the literature for inorganic iron were due to contamination with copper (see Hart and Elvehjem, 1927).

In 1944, Moore et al on the basis of experiments suggested that 1.5 - 10 times more ferrous iron is absorbed by human subjects than is the case when ferric salts are given. From these results, the theory arose that ferric might be reduced to ferrous form in the intestine and that this change was essential for absorption. Tompsett (1940) had suggested that iron might be reduced by food in the stomach and demonstrated that iron could be reduced in vitrio when treated with pepsin hydrochloric acid solutions. Bergeim and Kirch (1949) confirmed his findings and demonstrated that while 90 per cent of ferric iron in fresh vegetables was reduced, only 25 per cent of that in meat and eggs was so changed, while Sheets and Ward (1940) showed that legumes as compared to leafy vegetables had a 30 per cent higher availability of iron as judged by haemoglobin response in anaemic rats.

Thus a high iron content in the diet does not indicate an equivalent availability, which is further dependent upon a number of factors, the more well defined of which are indicated below. In addition, iron absorption is dependent upon a regulatory mechanism which controls the passage of iron across the mucosal cells of the intestinal tract in accordance with the needs of the body.

FACTORS AFFECTING ABSORPTION

Ascorbic Acid

It has been shown by Moore, Minnich and Welch (1939),

and Moore (1955) that oral administration of Vitamin C during feeding of iron salts, greatly increases iron absorption. Totterman (1949) has indicated however that a definite physiological relationship between ascorbic acid and iron metabolism is doubtful, and its effect on absorption is probably due to its reducing properties.

Phosphates

These compounds decrease iron absorption, the phosphate presumably combining with the iron and rendering it insoluble (Kinney, Hegsted and Finch, 1949).

Phytic Acid

Iron phytates are even less soluble than the phosphates, and McCance and Widdowson (1943) drew attention to the adverse effect of phytic acid on iron absorption. Walker, Fox and Irving (1948) have not been able to confirm these findings.

REGULATION OF ABSORPTION

Present theories of iron absorption are based on the idea put forward by McCance and Widdowson (1937) that there is negligible excretion of iron and absorption is controlled by body requirements. The mechanism regulating intake to limit total body iron must, however, be flexible to permit increased absorption should loss of iron occur, or the demands of the body for iron increase as in pregnancy. Such a mechanism was first demonstrated by Hahn et al (1939) using radioactive iron when they showed that whereas the normal dog absorbed negligible amounts, animals rendered anaemic by

bleeding absorbed the tagged salt and converted it quantitatively into haemoglobin in four to seven days. (Hahn et al 1940.) These conclusions assumed that absorption of the test dose was followed by its immediate and complete utilisation in haemoglobin synthesis. It was shown, however, by Greenberg et al (1947) and Dubach et al (1948) that this was not strictly true. By measuring unabsorbed iron in faeces after feeding tracer doses, Dubach and co-workers demonstrated that more iron was absorbed than could be accounted for in the RBC's.

The nature of the regulatory mechanism has been extensively studied by Granick and reviewed fully (1951). Hahn et al (1943) had previously ascribed this function to the mucosal cells of the gastro intestinal tract, postulating that an iron acceptor substance present passed from the intestine to the plasma, when iron level is low, or if plasma concentration is normal retains iron from the gut until saturation of the cell occurs. Granick (1946 a and b) considers the acceptor substance to be apoferritin.

As envisaged by Granick, when the mucosal cell is saturated as regards ferrous iron, equilibrium exists between ferric iron stored as ferritin and freshly absorbed ferrous iron; as the ferrous compound is removed by the serum, saturation is maintained by migration of iron from ferritin. When saturation can no longer be maintained by this means further absorption commences.

In 1939, Moore et al found that following oral loading with large amounts of iron, further absorption can occur as

is shown by a rise in serum iron; 50-100 mg of peroral iron being required. The increase in serum iron is roughly proportional to the quantity given up to a certain limit at which intestinal irritation becomes an affecting factor. However serum iron absorption curves are difficult to interpret, being influenced by such factors as rate of absorption and elimination from the blood stream, and the degree of saturation of the iron binding component of the plasma. Dubach and Moore (1948) feeding tracer iron showed that while subjects with hypochromic iron deficiency anaemia absorbed large quantities of iron, patients with refractory anaemia, untreated pernicious anaemia and haemochromatosis absorbed more iron that they were able to build into haemoglobin. However, in cases of hypochromic anaemia, previous injection of large amounts of iron decreased absorption from the intestine, although blood haemoglobin had not yet returned to normal.

The same workers employing dogs made chronically anaemic by repeated phlebotomy showed that the level of iron in the serum had no apparent effect on absorption.

That anaemia <u>per se</u> does not influence absorption was first suggested by Whipple and co-workers (1943), who found that in dogs rendered anaemic by repeated bleeding, increased absorption did not occur till storage iron fell, generally after a latent period of at least seven days. It would thus appear that, although the intestinal mucosa is one of the chief regulators of iron absorption, it is not so efficient

as was formerly supposed and absorption may occur without the need of the body for iron, yet the hypothesis offers the most satisfactory explanation of iron absorption in the light of present knowledge. As Granick (1954) has pointed out, blood oxygen tension can be correlated with an increased passage of iron from the mucosal cell into the blood stream, and it may be that an increased absorption in anaemia may be due to a diminution in oxygen getting to the mucosal cells or to the hormonal mechanism influencing iron intake.

EXCRETION

It was previously believed that iron was lost to the organism indiscriminately, but it is now realised that the body shows great economy in its handling of iron, only small amounts being excreted. This was first suggested by Whipple (1936) and by Widdowson and McCance (1937) who showed that loading iron stores by injection did not appreciably increase excretion, not did multiple blood transfusions (Widdowson and McCance 1943), and it became clear that as body iron did not increase indefinitely, absorption must be regulated.

Hynes (1948) estimated the daily loss of iron as 1.2 mg in man and 2.1 mg in woman. Moore (1955) arrived at a similar figure 1 - 1.5 mg, the chief loss being via the faeces, 0.3 - 0.5 mg and epithelial sweat 0.5 mg. In the adult female before the menopause with an average loss of some 35 - 70 ml of blood per menses the figure is twice that of the male.

In the mouse, Stevens et al (1953) found that the turnover of body iron was 0.5 per cent per day. Normal animals and animals with increased iron stores, regardless of sex, showed approximately the same loss of body iron per unit time. The larger domestic animal have not been investigated.

There is a constant small loss of iron in the urine which is unaffected by oral intake, but following intravenous injection a rise occurs in the ensuing twenty-four hours, about 1.5 per cent of the administered dose being lost in this way (McCance and Widdowson, 1938, Hahn et al, 1939, Vanotti, 1946). Cartwright et al (1954) give the mean urinary excretion of iron per twenty-four hours as 48µg. for man. This amount has been found to increase ten times in nephrosis coupled with a lowered plasma iron.

As all cells contain iron, a further constant small loss is due to epithelial desquamation, either of intestinal mucosa or epidermis, including hair.

Mitchell and Hamilton (1949) reported a daily loss of 6.5 mg due to sweating, but this was later disproved by Stewart et al (1950) using radioactive iron to eliminate epidermal contamination, and more recent work (Mitchell et al 1949, Johnston et al 1950) confirms the accepted figure of 0.1 to 0.2 mg per cent, while Adams (1950) found negligible amounts in sweat, 0.6µg. per 100 ml attributing the principle loss to desquamation.

Copp and Greenberg (1946) using Fe^{55} found no significant rise in biliary iron following injection. This had previously been demonstrated by Hawkins and Hahn (1944) who found that the daily basal excretion in dogs of 0.10 to 0.20 mg is maintained despite heavy oral and parenteral iron loading, but is increased by haemolysis (induced by phenylhydrazine), though even then, only some 3 per cent of the liberated iron is eliminated.

PLASMA IRON

Iron is transported in the body by the plasma and plasma iron is universally recognised as the fraction concerned in iron transport as distinct from haemoglobin. The level of plasma iron is dependent partly upon iron absorbed from the gastro intestinal tract, partly from that released through red cell destruction or required for haemoglobin synthesis, and is also dependent on the amount present in the storage depots of the body. In addition, an adequate amount of iron binding protein must be present. Thus changes in plasma iron values are representative of changes in metabolism.

Barkan (1927, 1933) showed that the iron of the plasma is non-dializable at pH 7.3 indicating that it is protein bound, and Valquist (1941) demonstrated by electrophoretic technique that the main part of the iron in serum had a mobility corresponding to the α and β globulins. Finally Schade and Caroline (1946) were able to show that iron was carried by the β globin fraction of the plasma. The specific iron binding protein, named siderophilin, has been

crystallised and found to have a molecular weight of about 88,000 (Laurell, 1947).

Laurell (1951) on consideration of available evidence has suggested that siderophilin serves as a carrier of iron in the same manner as haemoglobin is a carrier of oxygen, and that iron does not leave the blood stream as an iron globulin complex but in an ionised form. Schade, Reinhart and Levy (1949) have shown that combination of iron with siderophilin occurs in the presence of carbon dioxide, the iron being in the ferric state, and is dependent on pH, combination being at a maximum in neutral solution (pH < 6.5). When the pH becomes 5 or less, the whole of the iron is dialyzable. The iron is also removed by reduction to the ferrous state.

Normal plasma iron values vary between 118 µg and 143 µg per cent in males and between 98 µg and 123 µg per cent in females (table Ia). Ramsay (1953) removing plasma proteins by heat coagulation obtained a mean value of 171 µg/100 ml for males and 127 µg/100 ml for females. The reason for the sex difference is not fully understood, although depot iron is also greater in males. Cartwright and Wintrobe (1948) working with pigs claim that the serum iron level is affected by protein deficiency, plasma iron falling without saturation of the iron binding capacity occurring. Average values for animals are given in Table Ib.

Normally the binding component of the plasma, siderophilin is 33-39 per cent saturated (Rath and Finch 1949, Cartwright and Wintrobe 1949, Laurell 1947). Cartwright (1948) using protein deficient pigs, lowered plasma iron and the mean iron

binding capacity of the plasma, but the degree of saturation present in these animals was still one third. Laurell (1947) claims that the iron binding capacity of the plasma is increased in iron deficiency even though plasma iron is low. Following intravenous injection of excess amounts of iron, the resultant rise in plasma iron is to the level of the saturation value only. This 'braking effect' led Holmberg and Laurell (1945) to suggest that following saturation of the iron binding capacity of the serum, unbound iron rapidly leaves the blood stream and may be one of the factors associated with toxicity.

Following the work of Yuille et al (1950) who caused a fall in plasma iron following the production of sterile turpentine abscesses, Cartwright and co-workers were able to demonstrate that the level of plasma iron is lowered by agents causing a release of adrenal cortical steroids (turpentine, histamine, adrenaline, insulin and ACTH) and by cortisone itself. This has been shown in dogs (Hamilton et al 1951a) and in rats (Hamilton et al 1951b).

Where infective agents have been employed, Cartwright et al (1946), have shown that the hypoferraemia is combined with an accumulation of iron in the reticule endothelial system, and this gives an understanding of the low serum iron encountered during infections.

Plasma Iron Values in µg % in man and animals

Table Ia

Investigator	Man	Mean Value µg %	
		Male	Female
Fow weather	(1934)	125	105
Moore et al	(1937)	121	98
Valquist	(1941)	142	123
Powell	(1944)	143	117
Laurell	(1947)	124	108
Totterman	(1949)	140	121
Cartwright, Wintrobe	(1949)	127	123

Table Ib

Investigator	Animal	Mean Value µg %
Yuille et al (1950)	Dog	150
Campbell	Horse	170
Campbell	Ox	195
Cartwright, Wintrobe (1948)	Pig	169
Ramsay (1953)	Sheep	310

STORAGE

In determining body iron with the exception of red cell iron, any estimation of distribution is at best an approximation because of the difficulty of separating tissue from haemoglobin iron.

Figures for man (Granick 1954), and dog (Hahn 1937), are given in table II.

In adult man whose total body iron is some five grams, haemoglobin therefore represents 60-70 per cent. The iron content of the parenchymal cells, myoglobin, and haem enzymes, appears to remain relatively constant and cannot be utilised if iron loss should occur, and we find that the second largest fraction in the body is represented by available iron in the storage organs, the chief depots being liver, spleen, and bone marrow. (Bogniard and Whipple 1932; Hahn and Whipple 1936; Austoni and Greenberg 1940; Copp and Greenberg 1946)

Table II

IRON DISTRIBUTION

	Dog	Man
Blood Haemoglobin iron	57%	60-70%
Non-available iron:		
Myohaemoglobin	7%	3.5%
Parenchymal iron	16%	
Haem enzymes		0.2%
Available iron:		
Liver, spleen, bone marrow and other tissues	20%	15%

Depot iron occurs in two forms, ferritin and haemosiderin. Ferritin was first isolated by Laufberger in 1937, but has been extensively studied by Michaelis, Whipple and Granick. It consists of the protein apoferritin of molecular weight

460,000 and an iron containing portion of empirical formula $(FeOOH)_8$ $(FeOPO_3H_2)$, having magnetic properties which distinguish it from other ferric iron.

Ferritin contains up to 23 per cent of iron. Its function in the storage of absorbed inorganic iron and iron from destroyed RBC's was shown by Hahn, Bale, Granick and Michaelis (1943), while Granick and Hahn (1944) showed that 20 per cent of injected iron was converted into ferritin within two hours.

Haemosiderin is the term given to the granular pigment giving the histo-chemical reactions of iron and does not imply a definite chemical substance. When separated from horse spleen it may contain as much as 35 per cent of iron by dry weight. Haemosiderin is generally found in tissue that is high in ferritin.

There appear to be three sources of this substance, (a) as an intermediate product in the breakdown of haemoglobin, when it may be seen within the phagocytic cells of the reticulo endothelial system, (b) haemosiderin may be of intra-cellular origin - cytosiderin (Gillman and Gillman 1944), or (c) it may be formed from absorbed or injected iron (Hampton and Kahn 1953).

Rath and Finch (1948) consider that the degree of haemosiderosis of the bone marrow offers a rough estimate of general iron storage. None is found in iron deficiency anaemia in contrast to the massive deposits found in haemochromatosis. This was confirmed by Pratt and Johnson (1954).

Shodin, Gabrio and Finch (1953) from work on rabbits, suggest that at physiological levels, more ferritin than haemosiderin iron is present, but as the iron content of the storage organs rises haemosiderin predominates. At high levels additional storage iron is reflected by a quantitative increase in haemosiderin. When mobilisation of iron occurs, both the ferritin and haemosiderin fractions of the tissue are involved.

Hampton and Kahn (1953) using mice, found that during the first eleven hours after iron administration, the rate of haemosiderin formation predominated, but thereafter till forty-eight hours, the ratio ferritin/haemosiderin remained unchanged.

In a series of studies on storage iron in the dog, Finch et al (1950) found that of an average of 87 mgms of iron in the liver only some 5 mgms were not available for haemoglobin synthesis, confirming the suggestion of Bognaird and Whipple (1932).

Both age and sex appear to affect storage levels. Steenbock, Semb and Van Donk (1936) state that storage iron in the female rat exceeds that in the male. This was supported by Rose and Hubbell (1938) and by Widdowson and McCance (1948) who worked on rats, mice and hens but found the phenomenon was not present in the guinea pig and rabbit. At birth in all animals except the rabbit, liver iron represents only a small fraction of total body iron, but this increases greatly during suckling, except in the rabbit (McCance and Widdowson 1951), while piglets can only achieve

a similar quantity if an extra source of iron is given in addition to that supplied by the sow's milk.

In man a similar picture is found at birth but, in addition, a sudden increase occurs in the male about the twentieth year, and in females about the fiftieth year. Thus between puberty and the menopause storage in the male exceeds that in the female (Shairer and Rechenberger 1948).

Iron stores appear to accrue over a long period of time, and when depleted are replaced slowly, absorption in some way being regulated by the need of the body for iron, and in deficiency, once the needs of the marrow are satisfied accelerated absorption ceases and the reserves remain in a depleted state (Foutes and Thivolle 1936).

IRON UTILISATION

The total serum iron of man is about 4 mg, but it has been estimated that some 27 mg. of iron leave the blood stream daily, of which some 20 mg. are for haemoglobin synthesis (Huff et al 1950), the remainder presumably going to storage organs, chiefly the liver. Of this 27 mg, some 20 mg arise from red cell breakdown, the remainder from depot and absorbed iron. This suggests that iron from haemoglobin breakdown is rapidly reused while depot iron turnover is far slower (Greenberg and Wintrobe 1946). Huff et al (1950) estimate that less than one per cent of plasma iron turnover is concerned with absorption, and it does not appear that newly absorbed iron forms part of the 'labile iron pool' (West, Hahn et al 1952). This was first suggested by Copp and

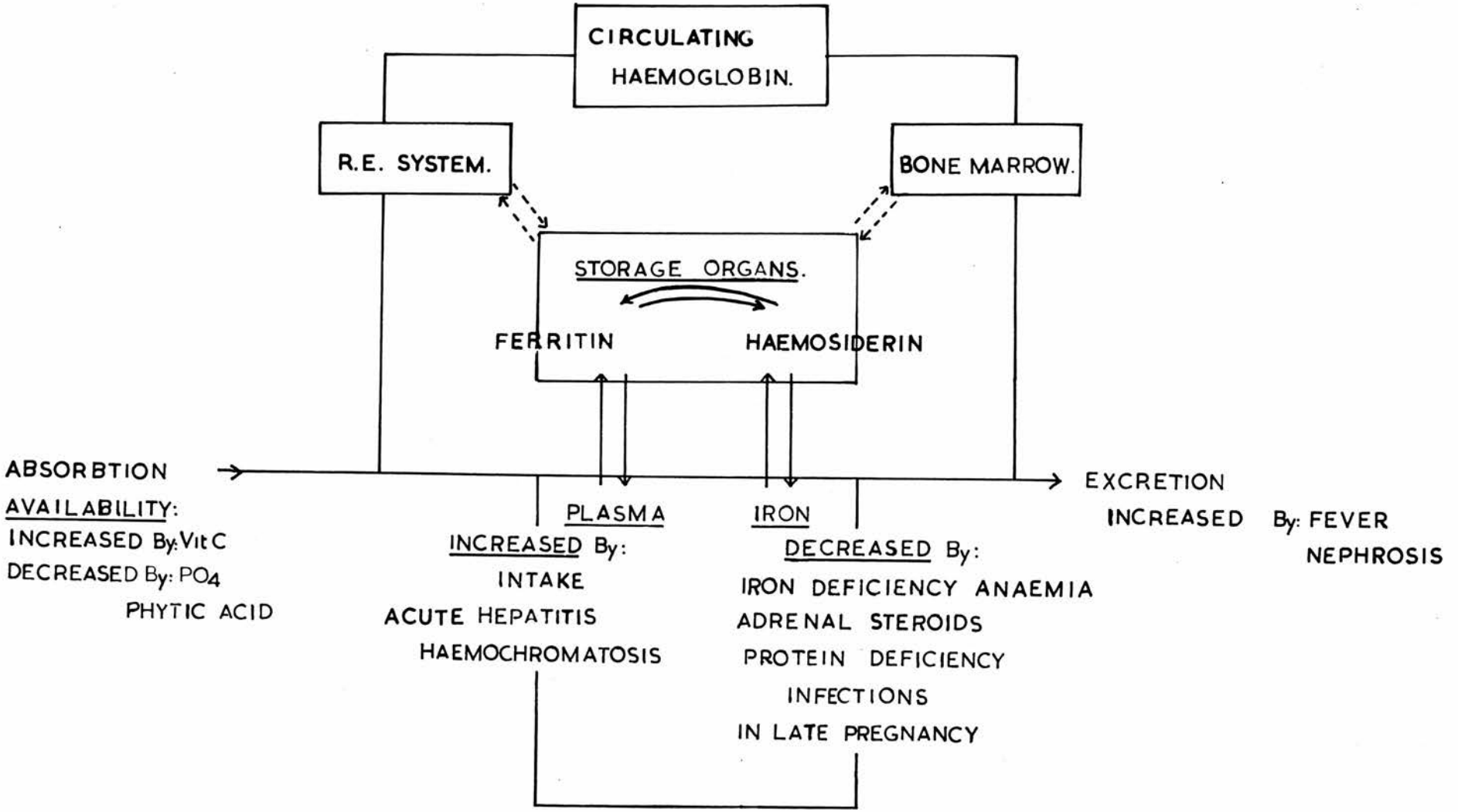
Greenberg (1946) who found that after feeding Fe^{55}, the amount present in the liver and spleen at first exceeded that in the blood, but the picture was later reversed, suggesting that some of this stored iron may have been used for red cell manufacture. Similar results were obtained following intravenous injection (Dubach, Moore and Minnich 1946), tagged RBC's beginning to appear in the circulation within twenty-four hours, but radioactivity did not rise to reach a plateau for two to three weeks.

Where the reserve iron store has been exhausted by repeated bleeding, Whipple and Robscheit-Robbins (1936) have shown that absorbed iron is almost quantitatively rapidly used for forming haemoglobin. Radioactive iron given orally appears in the red cells within four hours and is entirely converted into haemoglobin within four to seven days. (Hahn et al 1940).

Finch et al (1949) studied the effect of $Fe^{55:59}$ given intravenously in normal subjects and those suffering from various anaemias. Whereas utilisation in normal subjects averaged 74 per cent over eighteen days, subjects with iron deficiency anaemias showed rapid utilisation of the isotope for haemoglobin synthesis, while in haemochromatosis, refractory anaemias, and pernicious anaemia before therapy, where iron deficiency is not a causative factor, utilisation was markedly below normal.

The relationships in iron metabolism in the mammal are shown diagramatically in Fig. I.

FIG: I DIAGRAMATIC REPRESENTATION OF REGULATION OF IRON METABOLISM



THE DOMESTIC FOWL

In the chicken the study of iron metabolism has developed to a far lesser degree, the resulting picture being far from complete even in general outline.

The laying bird, unlike the mammal, in addition to meeting the needs of the tissue haem compounds and haemoglobin requirements of the blood, suffers a high loss of iron through the egg, and it was considered of great interest to determine how the chicken modified the accepted physiological mechanisms of iron metabolism to deal with this increased demand for iron upon the system.

HAEMOGLOBIN

The amount of haemoglobin in the blood of domestic chickens as observed from the literature is highly variable. While methods of determination may in part be responsible, it also appears that egg laying affects haemoglobin levels in the hen, marked differences being found between laying and non-laying birds (Cook and Harmon 1933; Maughan 1935; Tanaka and Rosenberg 1954). Olsen (1937) found seasonal variation to parallel the red cell count in male and female birds, the highest values being found in Spring, the lowest in Autumn.

Table III

Haemoglobin values (gms %) of normal chickens

Age	Male	Female	Method	Author
21 days	9.16	9.30	Modified Sahli	Holmes et al (1933)
42 days	9.7	9.6	"	"
63 days	9.7	9.7	"	"
84 days	10.1	9.7	"	"
Adult	13.5	9.8*	Acid haematin (corrected for turbidity)	Dukes and Schwarte (1931)
Adult	-	9*-14+	Dare	Cook and Harmon (1933)
Adult		8.9*	Modified Newcomer	Schultze and Elvehjem (1936)
Adult		7.4*	Dare	Harmon (1936)
Adult		10.95+	Dare	Harmon (1936)
Adult	13.94-9.9	10.6-7.55	Not stated	Olson (1937)
Adult		8*-8.1+	Fe estimation	Winters (1936)
Adult		8.9	Modified Newcomer	Surkie (1943)

* Laying. + Non-laying.

Cook and Harmon (1933) claimed haemoglobin fell some 40 per cent at the peak of laying. Maughan (1935) confirmed these findings noting a difference of 30 per cent in the level of laying and non-laying birds. Tanaka and Rosenberg (1954) blood sampled over 400 birds and found the differences in haemoglobin between laying and non-laying birds in a flock was not significant, findings similar to those of Winter (1936) and Schultze and Elvehjem (1936). However, in an individual study of 52 pullets while laying and non-laying, the differences were highly significant ($P > 0.01$) using the 't' test for paired data. These same workers found a negative correlation between haemoglobin and intensity of production of 0.3182, showing that good producers tended to have lower haemoglobin than poor producers.

Both Cook and Harmon (1933) and Tanaka and Rosenberg (1954) described an inverse relationship between haemoglobin and intensity of egg production. However, as non-layers have higher haemoglobin levels than layers, spasmodic bleeding of a whole flock would show a fall in haemoglobin inversely proportional to the number of eggs being laid. Further, despite a detailed study of 17 birds, Cook and Harmon were unable to show that length of laying affected haemoglobin levels.

The report of Shemin, London and Rittenberg (1948) that the mature avian erythrocyte continues to synthesize haemoglobin is of special interest, more so since Sharpe Krishnan and Klein (1951) have demonstrated uptake of iron

when added to duck red cells in vitro. The same workers, however, claimed a small uptake of iron by mammalian RBC under similar conditions. As this is in contradiction to the findings of Hahn et al (1940), confirmation of the findings of Sharpe and co-workers is awaited.

The life span of the avian red cell has been determined by Hevesy and Ottensen (1945) and Hevesy (1947) using radioactive phosphorous and by Rodnan, Ebaugh, and Spivey Fox (1951) with radioactive chromate. Both workers give alife span of approximately 28 days compared to the figure of 124 days for the dog proposed by Hawkins and Whipple (1938) who measured bilirubin excretion, and 120 days found by Shemin and Rittenberg,(1946) and Grinstein, Tower and Moore (1949) using radioactive glycine.

Besides sexual differences, age, as in mammals, appears to affect haemoglobin levels. Harmon (1936) reported that haemoglobin was high at hatching and lowest at two weeks of age, thereafter increasing in value till maturity was reached. Similar findings were reported by Holmes, Pigott and Campbell (1933) and Olson (1937). After twenty weeks of age the female level declines with onset of laying (Tanaka and Rosenberg 1954).

Storage

As in the mammal, both age and sex appear to affect storage levels of iron. Hegsted, Finch and Kinney (1952) give the mean quantity in 6 chicks as 2.31 mg per liver or 19.2 mg per 100 gm. Widdowson and McCance (1948) give the average figure for cockerels of twenty weeks of age as

8.7 mg/100 gm tissue and 14.7 mg/100 gm tissue for pullets. Chapman, Maw and Common (1950) showed that the weight of the liver can be increased 50 per cent by oestrogen treatment. At the same time the total amount of 'inorganic' iron in this organ increased a little, although the percentage figure fell.

The body distribution of iron in birds has not been determined. While iron can be demonstrated histochemically in bone marrow, the presence of ferritin has not yet been shown in either the liver or bowel epithlium.

In the present work it was found that while non haem iron could be shown to be present in the liver of laying hens by chemical means, none could be shown histochemically (ferrocyanide and HCl), and the possibility of a combination, such as with apoferritin, seems probable. As the effect of egg production, with accompanying loss of iron, on storage iron had not been studied, one of the objects of the present work was to investigate depot iron in immature laying and non-laying hens.

Facilities did not permit investigation of the influence of sex on iron reserves.

Utilisation and Excretion

In the laying bird which has a heavy loss of iron via the egg, these two subjects are probably best considered together. The amount of iron in the egg found by earlier workers has been reviewed by Needham. These results show

great variation, probably due to the analytical methods then available, and should be treated with reserve. Cruickshank (1941) quotes Hahman as finding an average value of 12.4 mg Fe/100 gm dried yolk. This would represent about one mg of iron per egg. Elvehjem and Hart (1929) give figures of from 0.8 mg to 1.0 mg, while Ramsay (1950) found an average of 1.06 mg per egg (range 0.64 to 1.72 mg). The same author (1951) reported average values of 1.23 mg per egg, but the eggs were larger and heavier than in his previous work. Schaible, Davidson and Bandemier (1944) give figures of 4.5 mg per egg, but this has not been confirmed. They also claim that the amount of iron in egg white increases during storage.

The amount excreted in the egg does not appear to be affected by increasing dietary iron (Elvehjem and Hart 1929; McFarlane et al 1930; Schaible et al 1944). The converse does not appear to have been investigated, probably owing to the difficulty of obtaining a diet adequate in all respects for egg production, but deficient in iron.

An attempt at depletion of iron reserves by bleeding was carried out during the present work in a few birds; one fifth of the estimated total blood volume being withdrawn, but no fall in egg iron, haemoglobin or laying performance was encountered over 17 days, and as no estimation of iron absorption over this period was possible the matter was not pursued further.

Warren and Conrad (1939) claim that the first yolk of a sequence is larger than succeeding ones, and depends on a difference in the period of growth or the amount of F.S.H.

liberated from the pituitary, and it was decided that account should be taken in the present work to see if the amount of iron in the yolk was affected this way.

The total serum iron in the chicken is unknown, but taking the plasma volume figures for hens of Sturkie and Newman (1951) (cf. Common, Bolton and Rutledge 1948 who used a washing out technique) and employing plasma iron values found in the present work, immature pullets and non-laying hens have 0.2 - 0.25 mg of total plasma iron and laying birds o.7 mg.

The shorter life of the avian erythrocyte necessitates a more rapid turnover of iron and the great loss while laying accentuates this. The finding of Shemin et al (1948) that the avian erythrocyte synthesize haemoglobin may indicate a different method of utilisation of depot iron to that accepted for the mammal, and the heavy loss while laying would suggest that a far greater fraction of plasma iron turnover in the avian is concerned with absorbed iron.

The absence of signs of clinical anaemia - unthriftiness, debility, scouring, or impaired appetite - in birds laying for long periods, suggest that either depot stores are extensive or recently acquired iron is rapidly utilised. As neither of these points has been investigated, studies were undertaken on utilisation of injected iron compounds and the resultant changes in storage iron.

Absorption

Before the present work was undertaken serum iron values in the hen had not been determined and no exact

knowledge of regulation of absorption of iron was possible.

Oral loading with large amounts of iron in the diet (2 per cent iron) will cause an increase in liver storage iron in chickens (Hegstead, Finch and Kinney 1952), a similar phenomenon being known in mammals (Kinney, Hegstead and Finch 1949), including man (Mitchell 1952).

The sites of absorption are unknown, but the mechanism must be capable of utilising a far greater proportion of available iron in the diet while laying to combat the heavy iron loss and the concept of availability obviously presents a different problem in this species.

The transport of iron had not been previously investigated and it was at once apparent that in a proper understanding of iron metabolism, plasma iron would play a major part and this aspect was studied in all experiments undertaken.

Programme of Investigation

To follow the normal pattern of iron metabolism, two groups of birds were studied over their respective laying seasons, and serial determinations of haemoglobin, serum iron, and egg iron, were undertaken throughout each period. The livers, spleens, and kidneys of the second group were analysed in addition, to follow the pattern of iron in the storage organs.

The remainder of the work was undertaken to follow the effect of induced alterations in the pattern of metabolism.

The fate of injected iron was first studied using ferrous ascorbate (Goetsh, Moore and Minnich 1946), in comparatively small quantity in the belief that transient changes would be produced. This work was followed by injection of relatively large amounts of saccharated oxide of iron (Cappel 1930, Slack and Wilkinson 1948) and it was expected that substantial increase in stored iron might lead to interesting effects.

Finally the demonstration by McCance and Widdowson (1948) that the livers of pullets contained more iron than of cockerels of the same age, and the finding of Chapman, Maw and Common (1950) that oestrogen besides increasing liver weight and size, also increased the amount of inorganic iron present, suggested that hormonal control may influence iron metabolism in birds. It was therefore decided to attempt to imitate the effects of laying by injection of oestrogenic substances into immature pullets.

Material and Methods

Birds

In the first experiment, sixteen brown leghorn pullets were utilised, and for the second season sixty birds in all were followed. All birds were maintained throughout in an intensive deep litter system on straw bedding. The buildings were centrally heated so that temperature changes were reduced to a minimum, and by this means the rate of egg production averaged 4-6 eggs per bird per week. All birds were trap-nested.

The diet consisted of commercial layers mash with an additional mineral supplement (Orr, Moir, Kinross, and Robertson 1925), the supplement allowing 1 mg of iron per bird daily, while the ration, assuming a hen eats 120 - 140 gms of food daily, contained 5 - 6 mg of iron.

In the first season, one bird became repeatedly crop bound, laid poorly, and was ultimately discarded. Of the sixty birds in the second group, two dozen of the poorer layers were rejected after a preliminary study of all birds over eight weeks at the commencement of laying. Of the thirty-six retained, one later developed marked ascites and was killed. This bird was excluded from the results. One bird developed a prolapse and was killed immediately; liver, spleem and kidney iron in this bird were determined and included in the final results.

Materials Examined

Haemoglobin and Plasma Iron

The birds were bled approximately every three weeks, commencing several days prior to laying; 5 ml of blood being withdrawn into a heparinised syringe from the wing vein. 0.1 ml duplicate samples were taken for haemoglobin determination and the remainder centrifuged at 3,500 r.p.m. for twenty minutes, when the supernatent plasma was decanted. 2.0 ml of this was then used to obtain plasma iron values.

Egg Iron

The eggs were collected daily, weighed individually and broken into a glass vessel. The empty shell was then weighed and the weight of the contents (yolk plus white) found by difference. In preliminary experiments treatment was then given in the glass homogeniser of Potter and Elvehjem (1936), for 5 minutes, to obtain a homogenous solution. 1 ml duplicate samples were then taken for determination of iron.

This method was very tedious and time-consuming and it was decided to bulk each bird's eggs at weekly intervals. However, such large amounts of solution could not be accommodated in the homogeniser. Because it was necessary to dilute the yolk before pipetting, the egg white was replaced with an equal volume of water. This could be evenly mixed with the yolk merely by stirring with a glass rod.

As a check on the validity of this simplified technique, a week's supply of eggs from the same hen were taken from time to time and homogenised individually as had been done previously. On no occasion was any marked change in weekly average iron values found.

Liver Spleen and Kidney Iron

With the second group of birds, analyses of carcases of several birds killed off after they had been in lay eight weeks showed that the bone marrow and muscle contained little iron, the principal deposits being in the liver, kidney and spleen, and it was consequently decided to restrict analyses to these organs.

At varying intervals throughout the laying season, groups of birds were killed off by serparation of the cervical vertebrae, allowing free bleeding. Organs were removed within an hour of death, weighed and mixed with nine times their weight of distilled water to give a final dilution of 1 in 10. This was then homogenised in the glass homogeniser of Potter and Elvehjem (1936) and the resulting homogenate gassed thoroughly with carbon monoxide, to abolish the interfering effect of haemoglobin (Ramsay and Campbell 1954).

The carbon monoxide was prepared by running concentrated sulphuric acid onto formic acid contained in a conical flask having a two-holed stopper. One hole carrying the delivery tube of a separating funnel containing the H_2SO_4, the other

having an outlet tap for controlling the flow of gas generated.

Methods of Iron Estimation

Total Blood Iron

The method of Ramsay (1952) was employed. Duplicate portions of 0.1 ml of blood were pippetted into tubes containing 1 ml of water and graduated at 12 ml and fitted with ground glass stoppers. After warming for one minute in a boiling water bath, 0.2 ml of hydrogen peroxide in acetate buffer (5 M acetate buffer pH4.6 and 10 vols hydrogen peroxide in equal vols) was added. After gentle shaking, heating was then continued for five minutes. After cooling, 1 ml of 40 per cent sodium sulphite, freshly prepared, was added, followed at the end of five minutes by 2 ml of 0.25 per cent $\alpha\alpha'$ dipyridyl in 50 per cent acetic acid ($^{v}/_{v}$). After shaking and further heating for ten minutes, the contents when cool were diluted to 12 ml with ethanol and shaken vigorously. After standing thirty minutes the contents were filtered through a No. 42 Whatman paper.

The optical density of the solution was then measured at 520 mμ in a Unicam SB350 spectrophotometer, a blank prepared similarly, being set at zero.

A standard graph was prepared by dissolving 0.146 gm of ferrous ammonium sulphate ($Fe(NH_4)_2SO_4$, $6H_2O$) in two litres of glass distilled water, and treating known aliquots (0.5 ml; 1 ml; 1.5 ml; etc.), as for the whole blood samples.

Plasma Iron

This was determined by the hydroxylaminedipyridyl method of Ramsay (1953, 1954) as this method gives higher values than those obtained by Kitzes Elvehjem and Schuette (1944), Tompsett (1934) and Fowweather (1934), who used HCl and trichloracetic acid extraction. Further, the procedure is less tedious and requires a smaller amount of plasma and the estimation is unaffected by slight haemolysis (Ramsay 1954).

To 2 ml of plasma in a test tube graduated at 7.5 ml was added 5 ml of acetate buffer 0.5 M. pH5, containing 0.075% $\alpha\alpha'$ dipyridyl and 0.1% hydroxylamine hydrochloride, followed by distilled water to the mark. After stirring and heating for five minutes in a water bath to coagulate protein, the contents were cooled and filtered through a No. 42 Whatman and read at 520 $m\mu$ as before.

Liver Spleen and Kidney Iron

Duplicate 1 ml alequots of homogenate were pipetted into tubes graduated at 7.5 ml, and 1 ml of 0.5 M sodium sulphate and 4 ml of 1 M acetate buffer pH4.25 containing 0.5% $\alpha\alpha$ dipyridyl, added. After heating in a water bath for thirty minutes the tubes were cooled, made up to the mark with glass distilled water and filtered via a No. 42 Whatman paper and read at 520 $m\mu$. It was found that if both tissue suspension and reagents were fully saturated with CO before mixing and heating, the interfering effect of haemoglobin was abolished, and if treatment was adequate

the precipitated protein remained pink throughout the experiment. A colour change to brown entailed repetition of the analysis, indicating possible interference by haem pigments or a slow rate of formation of the ferrous dipyridyl complex.

Egg Iron

This was determined by the hydroxlamine dipyridyl method of Ramsay (1951).

Duplicate 1 ml portions of the egg homogenate were pipetted dropwise (due to the viscosity of the mixture) into 12 ml graduated tubes. 4 ml of acetate buffer (1 M sodium acetate, 1.5 M acetic acid, 0.5% Hydroxylamine hydrochloride, 0.2% $\alpha\alpha'$ dipyridyl) were added and the tubes heated in a boiling water bath for ten minutes to reduce any complexes of iron present. After cooling and making up to the mark with ethanol, the tubes were shaken and, after standing thirty minutes, filtered through a No. 42 Whatman and the colour intensities read at 520 $m\mu$ as previously.

Iron values as before were interpolated from the standard graph.

EXPERIMENTAL RESULTS

Iron Distribution during the laying season

As the results obtained in each laying season were of a similar nature and varied only in the number of birds employed, no attempt has been made to present them separately. Liver spleen and kidney analysis were, however, only undertaken in the second period, when the larger number of birds employed permitted numbers to be killed.

Of the total of 50 birds studied, 14 were followed throughout laying till they went into the moult. Of these fourteen, 6 moulted in the second year and these were killed for analysis of depot iron. Twelve birds remained in full lay throughout the experiment, 7 in the first group, 5 in the second; the remainder of the second group being killed off in groups at fixed intervals, irrespective of length of time they had been laying.

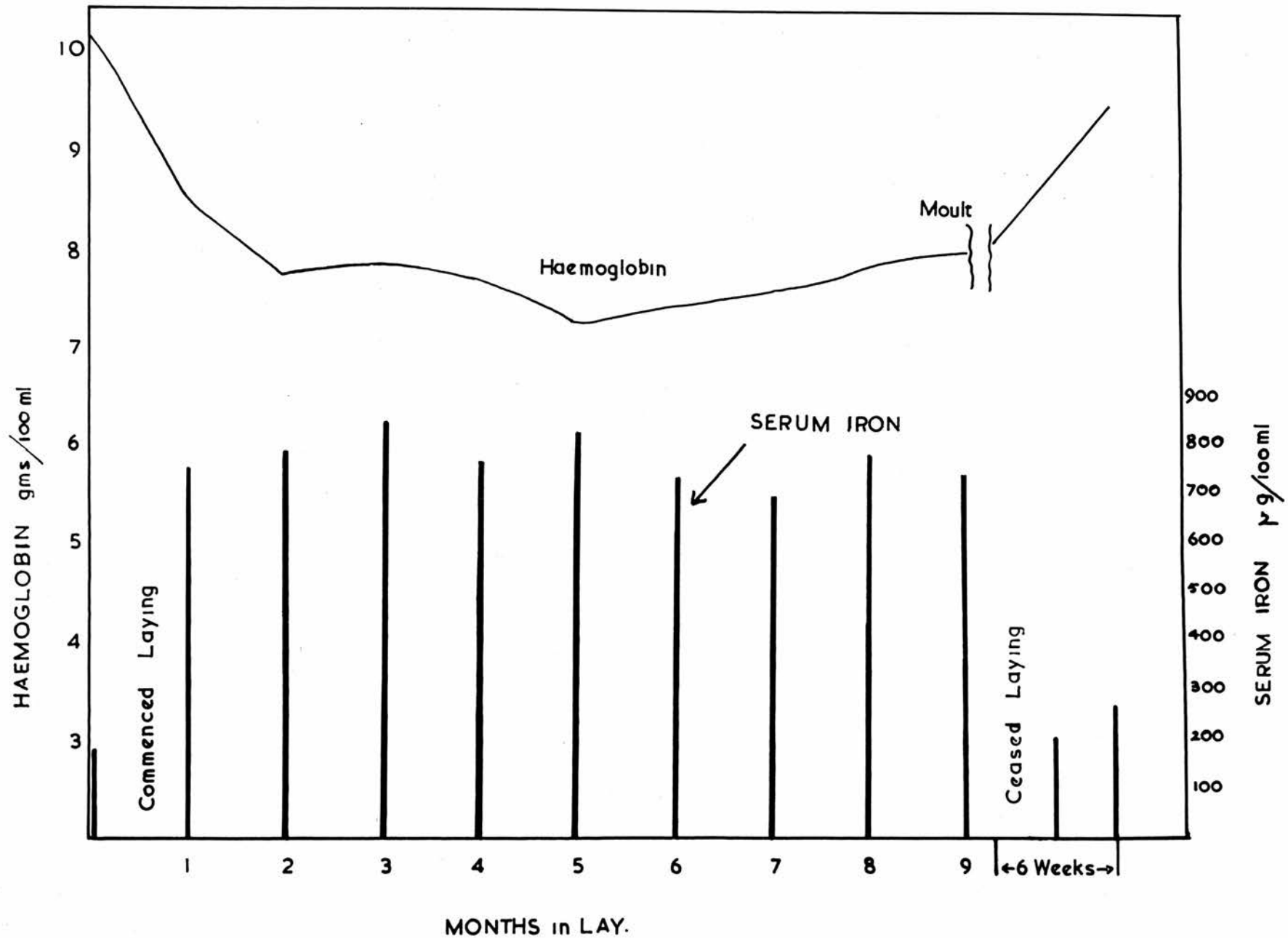
Haemoglobin

Mean blood haemoglobin concentration was calculated from total iron determination, assuming that fowl haemoglobin resembles many mammalian haemoglobins in containing 0.34% Fe (Bernhart and Skeggs 1943).

During laying a range of 6.5 to 9.1 gm Hb/100 ml was encountered, some birds having consistently higher values than others, although all birds were laying well.

FIG II

PLASMA IRON and HAEMOGLOBIN IN THE LAYING HEN



In cases where haemoglobin was determined at least fourteen days before onset of laying, a fall in values occurred with ovulation, the mean drop for thirteen birds being 2.2 gms, a value lower than that of Harmon (1933). Thereafter values remained fairly constant, fluctuating 5 - 10 per cent about a mean. Mean values for six typical birds followed throughout the season are given in fig. II. Some birds had ceased laying before they were killed and in these a rise towards the prelaying level was observed.

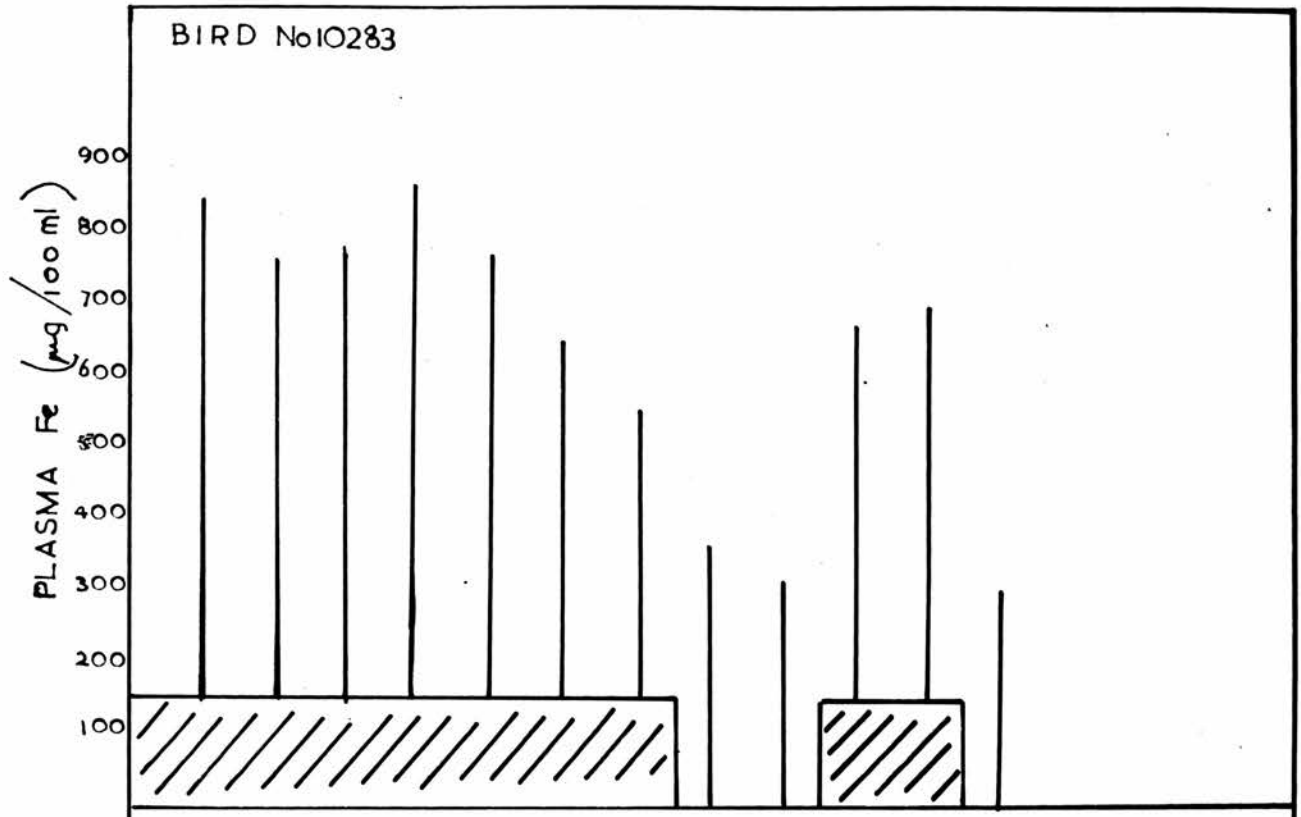
This rise was not apparent till egg production had ceased and was not observed in birds remaining in full lay. As only two pullets temporarily ceased production, no confirmation of the findings of Tanaka and Rosenberg (1954) that haemoglobin values started to rise two weeks before the laying pause was possible.

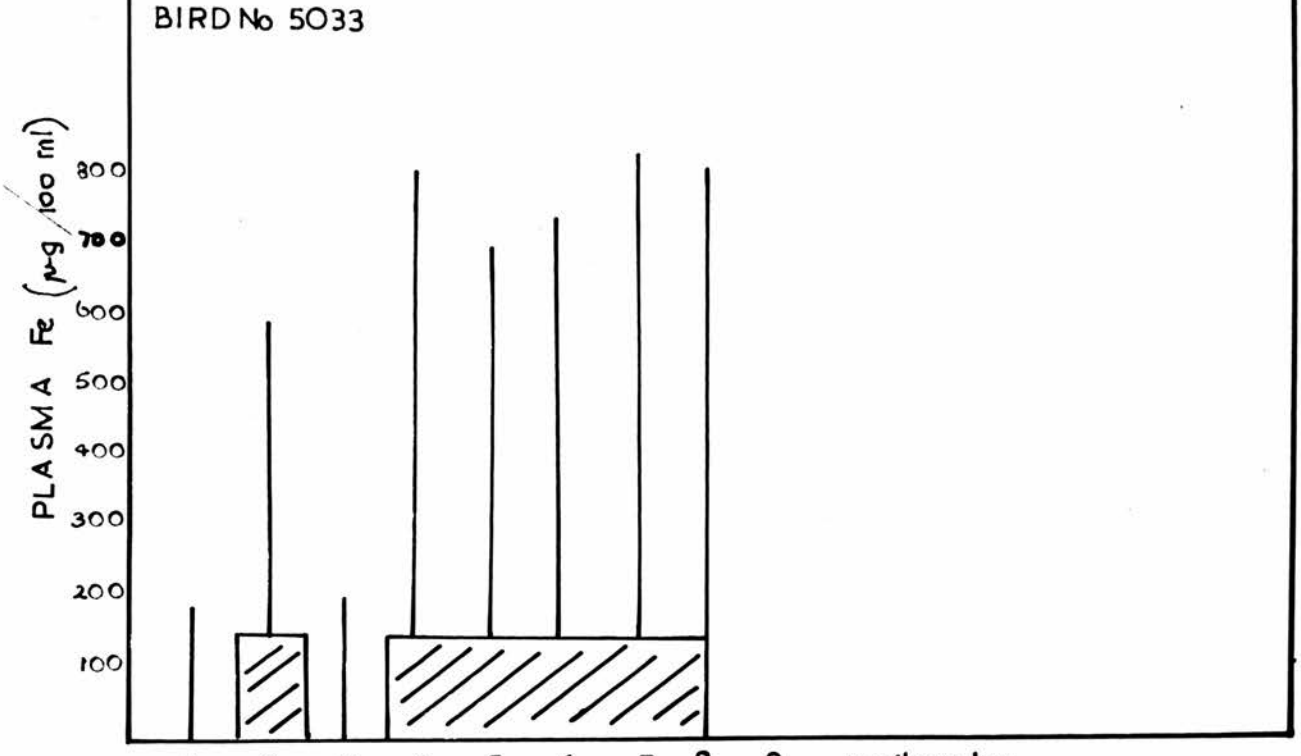
Plasma Iron

Up to about ten days before laying, plasma iron was found to lie between 100 and 250 μg/100 ml, a similar range to that found in other animals. Shortly before laying commenced it rose to 500 - 900 μg/100 ml and fluctuated within these high limits as long as eggs were produced. Cessation of laying brought a return to the original low level, fig. II. A temporary cessation of egg production in two birds produced a similar picture, fig. III.

FIG III.

RELATIONSHIP BETWEEN PLASMA IRON AND EGG PRODUCTION.





Shaded areas represent laying periods.

Egg Iron

The eggs laid in the first season tended to be lighter in weight than those laid in the second, the average iron content being 0.88 mg/egg (range 0.76 - 1.07 mg), compared with 0.94 mg/egg (range 0.76 - 1.27 mg) for the larger group of birds. In all some 7,000 eggs were bulked and analysed at weekly intervals.

At the commencement of laying, iron values tended to be some 30 per cent lower than average, but within fourteen days this was no longer apparent. Warren and Conrad (1939) claimed that the first yolk of a sequence or clutch is larger than the succeeding ones, but in the present work, when eggs were homogenised individually, no significant fall in the amount of iron could be detected in separate eggs of a clutch and mean values for any one clutch did not differ significantly from the general mean value. Table IV gives an illustration of typical values obtained.

Table IV

Relation of Egg Iron and Egg Weight to Sequence in a clutch

Clutch No.	Egg Iron	Egg Wt.	Clutch No.	Egg Fe	Egg Wt.
1	800 gm	42.3 gm	2	995	51.1 gm
	915 "	42.6 "		795 "	40.7 "
	762 "	43.5 "		935 "	42.5 "
3	870 "	45.7 "	4	903 "	43.0 "
	915 "	43.7 "		856 "	42.8 "
	858 "	42.9 "		850 "	40.8 "
	836 "	41.9 "			
5	860 "	44.9 "			
	1020 "	45.3 "			
	970 "	43.9 "			
	920 "	44.2 "			

This hen L3221 laid in clutches of 3 or 4 with a day between clutches.

No relation could be shown between egg iron content and number of eggs laid or length of time a bird had been laying.

Further, at cessation of laying or if a bird temporarily ceased to lay, no decline in the amount of iron present in the egg was found beforehand.

Liver Spleen and Kidney Iron

Table V shows that per 100 gm tissue the spleen possesses the greatest concentrations of iron, but the weight of this organ is only about one gram. The great bulk of stored iron in the hen appears to be present in the liver which contains 8 - 10 times as much non haem iron as the kidney.

The relation found between non haem iron and ovarian development is shown in Table VI. The livers of non-laying pullets contain an average of 7.7 mg non haem iron. In laying birds the amount was considerably less but reverted towards the prelaying figure on cessation of egg production (Table VII). While the difference in non haem iron between immature and laying birds was significant, no correlation could be found between the amount of non haem iron present and length of laying period and, although the six birds which ceased laying showed a higher mean non haem iron, the rise was again not satistically significant due to the wide variation in amounts of iron present and the small numbers ceasing production.

Table V

Amount of Non Haem Iron present in Liver, Kidney and Spleen of birds killed after stated periods of egg production

Bird No.	Weeks of Laying	Liver Wt. (gms)	Liver Non Haem Iron (mgm)	Non Haem Fe (mgm per 100 gms tissue)			
				Liver	Kidney	Spleen	Remarks
10272	6	41.3	3.39	8.18	10	6.99	Laying when killed
9789	11	45.73	5.66	12.4	12.3	16.2	"
9735	11	42.07	4.2	10	8.67	10	"
10293	11	42.67	3.07	7.03	10	15.6	"
9763	12	35.46	2.73	7.7	7.93	13.6	"
9696	12	42.16	2.31	5.47	5.19	11.7	"
9744	13	44.47	4.22	9.51	14.2	20	"
10063	14	34.03	4.14	12.2	6.39	10	"
10317	14	36.27	2.43	6.7	4.99	7.22	"
10202	15	40.03	2.49	6.0	3.95	10	"
10470	16	44.87	3.58	7.99	4.33	10	"
9689	17	29.4	1.94	6.6	2.6	6.44	"
10295	19	27.53	3.3	12	5.28	16.6	"
9803	19	42.1	2.59	6.19	4.75	9.1	"
10041	20	55.17	3.53	6.4	5.29	18.1	"
10258	23	33.78	2.02	5.98	6.61	8.77	"
9754	25	38.7	2.21	5.71	5.0	14.2	"
10316	26	37.93	1.9	5.0	4.0	10.5	"
10056	37	32.57	3.8	11.9	3.81	8.44	Ceased laying
9793	38	30.97	3.91	10.0	5.0	9.68	"

Table V (continued)

Amount of Non Haem Iron present in Liver, Kidney and Spleen of birds killed after stated periods of egg production

Bird No.	Weeks of Laying	Liver Wt. (gms)	Liver Non Haem Iron (mgm)	Non Haem Fe (mgm per 100 gms tissue)			
				Liver	Kidney	Spleen	Remarks
10330	39	47.5	9.5	20.0	6.21	11.7	Ceased laying
10238	40	55.13	7.42	13.4	4.44	11.02	"
10291	43	35.66	5.99	16.7	6.59	11.9	Laying when killed
10299	43	27.94	7.88	28.2	5	8.9	Ceased laying
9687	44	51.09	3.71	7.26	6.32	10.3	Laying when killed
9756	44	22.62	2.03	11.3	3.1	10.7	"
10283	44	28.71	3.6	12.5	5.5	15	Ceased laying
1127	45	36.02	3.38	9.38	5.4	8.42	Laying when killed

Table VI

Plasma, Liver, Spleen and Kidney Fe in hens during development of ovarian activity
(Figures in parenthesis indicate the range of the individual observations)

Group	State of Ovarian Tract	No. birds	Plasma Fe (µg/100 ml) non haem Iron	Liver Fe (mg/organ) non haem Iron	Kidney Fe (mg/organ) non haem Iron	Spleen Fe (mg/organ) non haem Iron
A	Immature	7	225	7.7	0.70	0.20
			(173 - 290)	(6.3 - 10.5)	(0.57 - 1.03)	(0.13 - 0.22)
B	A few yolks up to 6 mm diam.	5	311	6.1	0.59	0.20
			(225 - 565)	(3.9 - 7.2)	(0.41 - 0.81)	(0.11 - 0.36)
C	Several yolks up to 15 mm diam.	6	438	6.7	0.90	0.16
			(318 - 559)	(4.4 - 8.2)	(0.44 - 1.38)	(0.14 - 0.19)
D	First egg acquiring shell	1	711	9.2	0.70	0.22
E	Laying	5	768	5.7	0.82	0.14
			(670 - 942)	(4.1 - 7.9)	(0.54 - 1.09)	(0.12 - 0.16)

Table VII

Plasma Iron, Liver Iron, and Liver Weight in laying hens
(Figures in parenthesis indicate range of individual observations)

Weeks in Lay	No. of birds	Plasma Iron (µg per 100 ml)	Non Haem Liver Iron (mg per organ)	Liver Weight (gm)
6	1	850	3.39	41.3
12	5	765 (555 - 850)	3.59 (2.31 - 5.66)	41.1 (35.7 - 45.46)
18	6	718 (565 - 915)	3.1 (1.94 - 4.22)	38 (29.4 - 44.8)
24	5	761 (630 - 870)	2.73 (2.02 - 3.5)	39 (27.5 - 55.2)
44	4	666 (565 - 755)	3.04 (2.03 - 5.99)	36 (22.6 - 51.0)
Ceased Laying	6	242 (175 - 301)	6.03 (3.6 - 9.5)	36 (27.9 - 55.1)

The Storage and Utilisation of Iron Administered Parenterally to Laying Birds

Introduction

The previous work demonstrated that during laying the hen had a high daily loss of iron for several months. Despite this, plasma iron remained high and, although haemoglobin continued at a low level, iron stores did not fall to zero. However, not all the iron in the storage depots is equally accessible for haemoglobin synthesis; some 5 per cent of total body iron, according to Bogniard and Whipple (1932), is wholly unavailable for red cell manufacture. It was therefore physiologically possible that depot iron might, while laying, be at the irreducible minimum for normal well being; gut absoption at its maximum being just sufficient.

When intake of iron is raised in normal subjects, an absolute increase in body iron stores ensues (Finch et al 1950), since iron is essentially a 'one way substance', with little excretion. In subjects with iron deficiency anaemia, radioactive studies indicate that administered iron is rapidly used for haemoglobin synthesis (Hahn et al 1940). Although subjects with depleted iron stores will more readily absorb iron from the gut, normal subjects require special diets which are generally low in phosphate to achieve increased iron uptake by the oral route (Kinney et al 1949; Finch et al 1950). It has been shown by Finch et al(1950) that distribution of iron in normal dogs following increased oral absorption was identical with that achieved by parenteral administration. As the latter method permits accurate

knowledge of the amount of iron received, requires no special diets and is easily performed, it was decided to study the effect of parenteral iron administration in hens.

In the mammal, the results of parenteral injection depend on the form in which the iron is administered, the total amount given, and the time interval between injection and examination. Subcutaneous or intramuscular injections are rarely employed in clinical medicine due to the severe local reaction encountered (Nissen 1947). This is not so apparent with animals, but focal inflammation and slow absorption from the injection site render the method unsuitable for experimental work.

Iron compounds which may be administered intravenously can be divided into easily and poorly dissociable compounds. The injection of the easily dissociable forms, which include such compounds as ferrous chloride and ferric ammonium citrate, results in severe toxic reactions as soon as an amount exceeding the iron binding capacity of the plasma has been given. The problem has been investigated by Polsen (1928), Holmberg and Laurell (1945), Laurell (1946), Somers (1947) and Slack and Wilkinson (1949) and appears to depend on the fact that simple ionic compounds cause immediate protein coagulation. The poorly dissociable iron compounds such as ferrous ascorbate and saccharated oxide of iron are far better tolerated; as much as several hundred milligrams of saccharated iron may be given in a single injection (Nissen 1947, Slack and Wilkinson 1949). Finch et al (1950), in a study extending over two years, claimed that iron from

ferrous ascorbate remained largely in the reticulo endothelial system in preference to parenchymal tissue. Polsen (1928) and Cappel (1930) who used saccharated iron suggested that the iron was transferred from the Kupffer cells to the polygonal cells of the liver.

In the chick, heavy loading with oral iron (2% of the diet) has resulted in increased liver iron (Hegsted, Finch and Kinney 1952), but no work on parenteral iron administration has been reported. It was therefore agreed to observe the effects of intravenous injection of small and large quantities of iron in the laying bird.

Accordingly the two methods selected were:

(a) Injection of 3 mg of neutralised ferrous ascorbate in 0.5 cc solution.

(b) Injection of 20 mg of saccharated oxide of iron in 0.5 cc solution.

The birds were housed, fed and trap-nested as before and observed for at least fourteen days before injection to ensure that they were laying well.

Ferrous Ascorbate

Procedure

In all twelve birds were used, three of which were employed as controls. All birds were bled seven days before injection and again just prior to the intravenous administration of iron. This was achieved by allowing the needle to remain in the vein after withdrawal of the sample to be analysed, and then attaching a tuberculin syringe containing 3 mg of ferrous ascorbate in 0.5 ml solution to the needle. The time taken for the injection was approximately one minute.

The experiment was run in two parts, six birds being used each time. The second part was identical with the first and was employed as confirmation. When injecting the first birds an unneutralised preparation was used. The birds showed evidence of distress and two collapsed. The signs were those of acute shock. Recovery took about ten minutes.

When the work was repeated, a neutralised preparation was employed and no untoward results were observed.

Haemoglobin and Plasma Iron

The controls were bled at weekly intervals. The remaining nine, after injection, were bled at intervals of one, three, seven, and fourteen days. It would have been of interest to measure the plasma iron frequently during the first few hours. For technical reasons this was not possible and the first specimen was, therefore, taken only after the immediate effects of the injection had subsided.

Results

Plasma Iron

The 3 mg of ferrous ascorbate injected was not reflected in the level of plasma iron at the end of 24 hours, nor in serum levels in the 14 days following injection (Table VIII).

Table VIII

Changes in Plasma Iron following injection of 3 mg of Ferrous Ascorbate

	Days Before Injection		Days after Injection			
Bird No.	7	0	1	3	7	14
	Plasma Fe µg/100 ml		Plasma Fe µg/100 ml			
9928	735	560	515	630	780	600
9946	700	545	630	885	715	635
9949	675	640	639	570	450	570
9938	500	670	545	710	710	920
9937	870	750	470	550	820	630
9940	770	540	470	490	350	180*
9948	640	470	530	590	560	460
9941	735	610	690	715	650	800
9694	837	700	790	760	920	1150
Controls						
261	625	635	670	705	850	730
9945	800	550	460	710	760	660
226	837	700	700	736	815	685

*Ceased Laying

One bird (9940) which ceased laying on the third day following injection, maintained the typical fall in plasma iron seen at cessation of ovulation suggesting that the excess iron must have been taken up rapidly by the storage organs. About 50 per cent of the birds showed a slight fall in serum iron at the end of twenty-four hours, although these lowered values were still within the range of plasma iron during laying.

The actual injection is certain to have produced a very marked increase in plasma iron, and it would have been of great interest to have followed the disappearance of excess iron from the plasma. This, however, would have required an acute experiment, and it would have been impossible to examine the effect of the injection on egg iron.

Haemoglobin

It would have been expected that the lowered haemoglobin found in laying birds might have resulted in utilisation of the additional iron by the bone-marrow for red cell synthesis. However, over the fourteen days following injection no significant change in haemoglobin values was encountered (Table IX).

Table IX

Blood Haemoglobin (g/100 ml) following injection of 3 mg of Ferrous Ascorbate

	Days Before Injection		Days after Injection			
Bird No.	7	0	1	3	7	14
	Hb 9/100 ml		Hb 9/100 ml			
9928	8.45	9.35	9.4	8.7	8.3	8.7
9946	9.2	7.8	8.2	8.1	8.45	8.1
9949	9.2	8.7	8.2	8.6	8.1	7.7
9938	9.0	8.2	8.1	8.7	8.4	8.1
9937	8.6	9.2	8.7	8.2	8.2	8.9
9940	8.4	7.9	9.2	9.9	10.4	9.9
9948	8.7	8.2	8.95	8.1	8.0	8.45
9941	8.3	8.1	7.7	8.2	7.9	8.3
9694	7.6	7.2	7.85	7.6	7.6	7.2
Controls						
261	7.7	7.9	8.1	7.4	7.8	8.6
9945	9.2	8.25	8.1	8.4	8.1	8.6
226	7.9	8.1	7.7	7.3	7.8	7.4

Egg Iron

Analysis of egg iron from all birds showed no significant rise following injection and figures from four typical birds and two controls are given in table X. Any increase in iron content did not exceed ten per cent of the previous values and such fluctuations are typically encountered during laying. As eggs are laid in clutches, a series of rapidly developing highly vascular follicles were present in the ovary at the

time of injection. The absence of a marked increase in iron content of the eggs laid following injection indicates no ready uptake of injected iron from the plasma by the follicles despite the marked rise which must have occurred in plasma iron.

Table X

Iron in Eggs before and after Injection

	Bird No.	9928	9946	9949	9938	Controls 261	Controls 9945
	Date	Fe(μg)	Fe(μg)	Fe(μg)	Fe(μg)	Fe(μg)	Fe(μg)
Days before injection	7		1015		1105	1305	
	6	1025	975	1090		1180	1020
	5	940		1100	1020	1080	985
	4	1110	965		1080		1110
	3		1045	1120		1260	1150
	2	1070	995	995	1120		
	1	1090		1080	1100	1375	1040
Days after injection	Inj.		1040	885		1155	
	2			940	1030	1300	960
	3	1110	1100	970	1160	1150	1055
	4	1110			1055		1130
	5		1000	1000	1020	1305	
	6	1130	1020	1090		1135	1185
	7			1130	1080	1130	1120
	8		1000	1210	1090		1160
	9	1145	1000	1155			1130

Saccharated Oxide of Iron

Method and General

Twenty-four pullets were used. They had been laying for several months and included, as a matter of interest, two birds which had gone into the moult. Management and housing of the birds were as before.

Seven days prior to injection, the collection of eggs was commenced and the first blood sample taken. At the end of the week, 20 mg of iron in the form of the saccharated oxide having a volume of 0.5 ml was injected slowly into the vein, after the second blood sample had been withdrawn as in the previous experiment. No untoward results were observed following injection.

The experiment was carried out in two parts, the second part extending and confirming the first in which six injected birds and three controls were employed.

Plasma Iron and Haemoglobin

This was determined in all birds seven days prior to injection and again just previous to the administration of iron. Thereafter, blood was collected at seven day intervals (to reduce likelihood of haematoma) unless birds were sacrificed between these dates when blood was taken for analysis.

The method of analysis was as before.

Egg Iron

Each egg was homogenised separately in a glass homogeniser. The previous method of analysis was employed,

except that, in addition to the fifteen minute heating, separate duplicate samples were heated for one hour to allow determination of any saccharated iron which had been taken up unchanged by the eggs (Ramsey 1954). Treatment in all other respects was exactly as before.

Liver, Kidney and Spleen

The organs were all removed within two hours of death, weighed, diluted one in ten and homogenised as previously. The method of analysis was as before.

Results

Plasma Iron and Haemoglobin

Following injection, birds were killed on first, third, seventh, fourteenth, twenty-first and twenty-seventh days, and blood values obtained on these dates in addition to the usual seven day intervals are shown in Table XI for fifteen birds.

A marked elevation in plasma iron, present at twenty-four hours, was still apparent after three days, but had declined by the seventh day. In the two non-laying hens a heightened plasma iron was still apparent after seven days when they were killed. One bird (4732) laying in the week prior to injection and with correspondingly high serum iron at that date was found to have no developed yolks (over 0.5 cm in diameter) in the ovary when killed after three days and serum iron figures had dropped to the non-laying level despite injection.

Table XI

Plasma Iron in µg/100 ml

Bird No.		4060	4995	9940	4107	4363	4732	9948	4061	5722	5735	5754	5020	5718	5004	613
Days before injection	7	480	470	520	580	625	500	560	570	600	535	725	555	830	200	200
	0	500	450	480	508	625	550	580	510	710	600	770	610	990	185	200
Days after injection	1	625	730	680												
	3				535	720	210									
	7						*	650	620	570	540	985	730	890	270	220
	14									750	620	830	560	930		
	20									500	570	800		580	Non Laying Birds	
	27											750		650		

*Stopped laying

In all birds, haemoglobin during this period showed no significant increase over pre-injection levels.

Egg Iron

Values for egg iron fluctuated within normal limits prior to injection. Eggs laid within twenty-four hours following injection would probably already be in the oviduct when iron was administered. Such eggs showed no increase in iron content by the usual method of analysis, but on prolonged heating to detect iron in the saccharated form increased values were obtained. As the exact time of ovulation and laying were not known, and as the whole egg had been homogenised, it was not possible to say whether the injected iron was in the yolk or had been excreted by the oviduct with the albumen.

Eggs laid within three days of injection had increased amounts of iron present, but once plasma iron levels had reverted to pre-injection values no increase in egg iron was encountered. However, if samples were submitted to heating for an hour, increased iron values were obtained demonstrating uptake of the saccharated iron by the yolks. Figures for five typical birds are given in Table XII. This uptake of iron in the injected form was found in eggs laid over 7 - 9 days following iron administration, implying that all developing yolks in the ovary were involved. Confirmation was obtained by analysing yolks above 1 gm in weight in the three pullets killed on the day after injection. Results are given in Table XIII and show that saccharated iron was present in all ova. The amount taken up appeared to be independent of the size of the yolk. Ova from birds killed at later dates showed a similar phenomenon.

Table XII

Bird No.	5718		5722		5020		4061		5735	
	Fe in μ gms per egg		Fe in μg per egg		Fe in μg per egg		Fe in μg per egg		Fe in μg per egg	
Eggs Laid										
9	1340		1075							
8	1250		1070		1050		830		1500	
7	1150		1160		1010		805		1460	
6	1090								1340	
5	950		875		1160		895			
4	1090		970		960		820		1340	
3	1000		1025				83		1160	
2	1010		990		1070				1210	
1	985		970		1040		850		1340	
0			800		1190		840		1370	
Days before injection										
1			970	1440						
2			1120	1605			1010	1320	1350	1700
3	1555	1840	1175	1660	1360	1590			1260	1800
4	1460	1860	1300	1820			775	830	1335	1665
5	1120	1840	1220	1860			890	1150		
6	1040	1265	920	960						
7	1130	1220	915	960	1185	1185	810	810	1540	1860
8	980	1120	820	835					1400	1400
9	1150	1160	900		1220				1360	
10	1160	1160	915		1040				1290	
11	1225		1005		980		Killed after 7 days			
Days after injection		Heated for 1 hr.		Heated for 1 hr.		Heated for 1 hr.		Heated for 1 hr.		Heated for 1 hr.

Table XIII

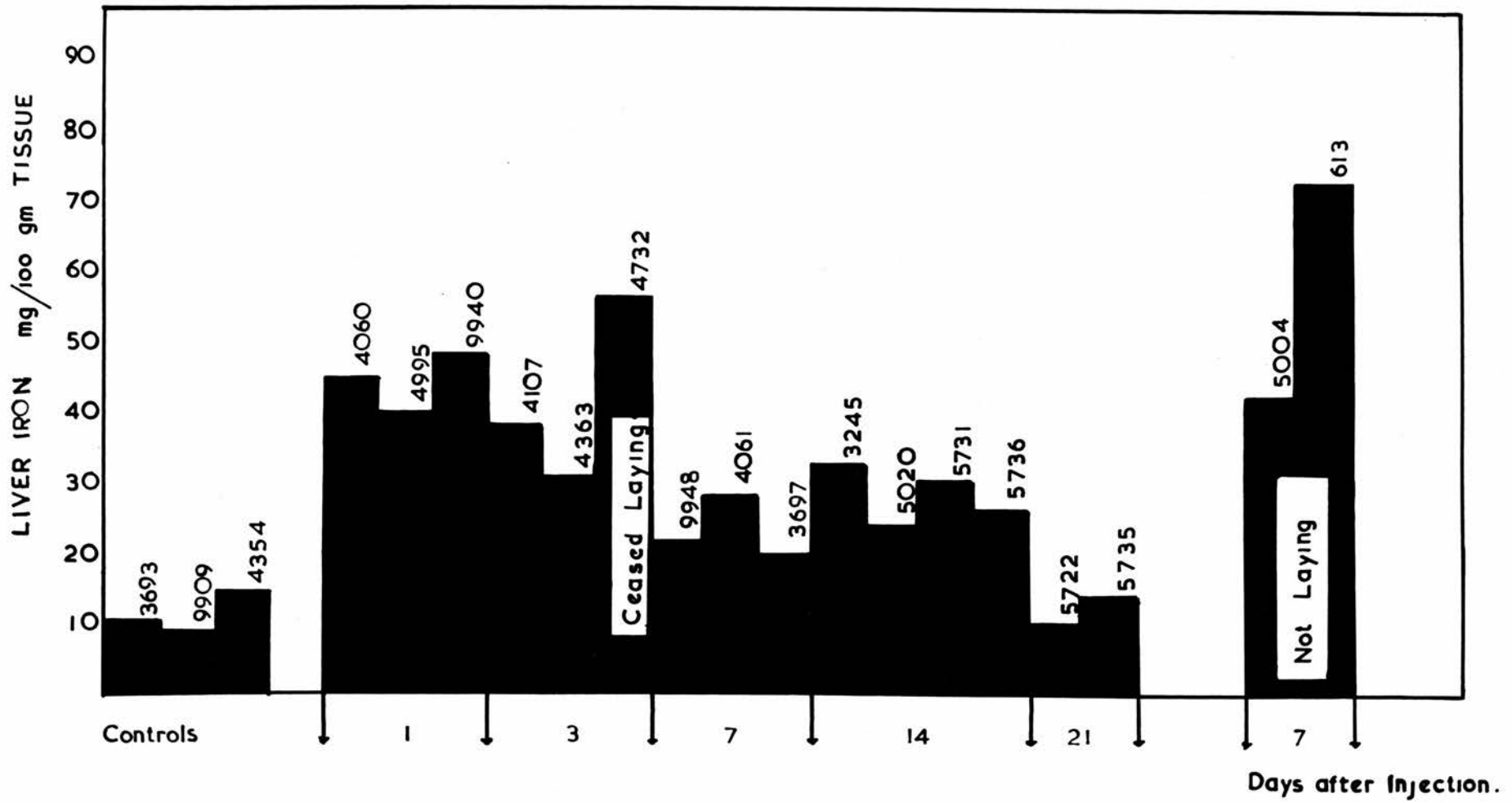
Iron in Unlaid Eggs μg per egg yolk

Bird No.	4995		4060		9940	
		After heating 1 hr.		After heating 1 hr.		After heating 1 hr.
	590	636	650	690	910	910
	570	800	654	790	810	950
	280	424	450	704	328	494
			210	358		

FIG V

CONCENTRATION OF NON HAEM IRON IN LIVER

LIVER IRON PER 100gm TISSUE



Liver Iron

In the three control birds liver non haem iron values were in agreement with results in the earlier work.

After injection the amount present increased in all cases, the quantity found declining rapidly with length of time between injection and death. Sections of the livers of some of the injected birds were made and stained with Perle's stain (sodium ferrocyanide and hydrochloric acid), to demonstrate iron deposits, but without success, probably due to the fact that these birds were not killed till fourteen days after the injection of saccharated iron.

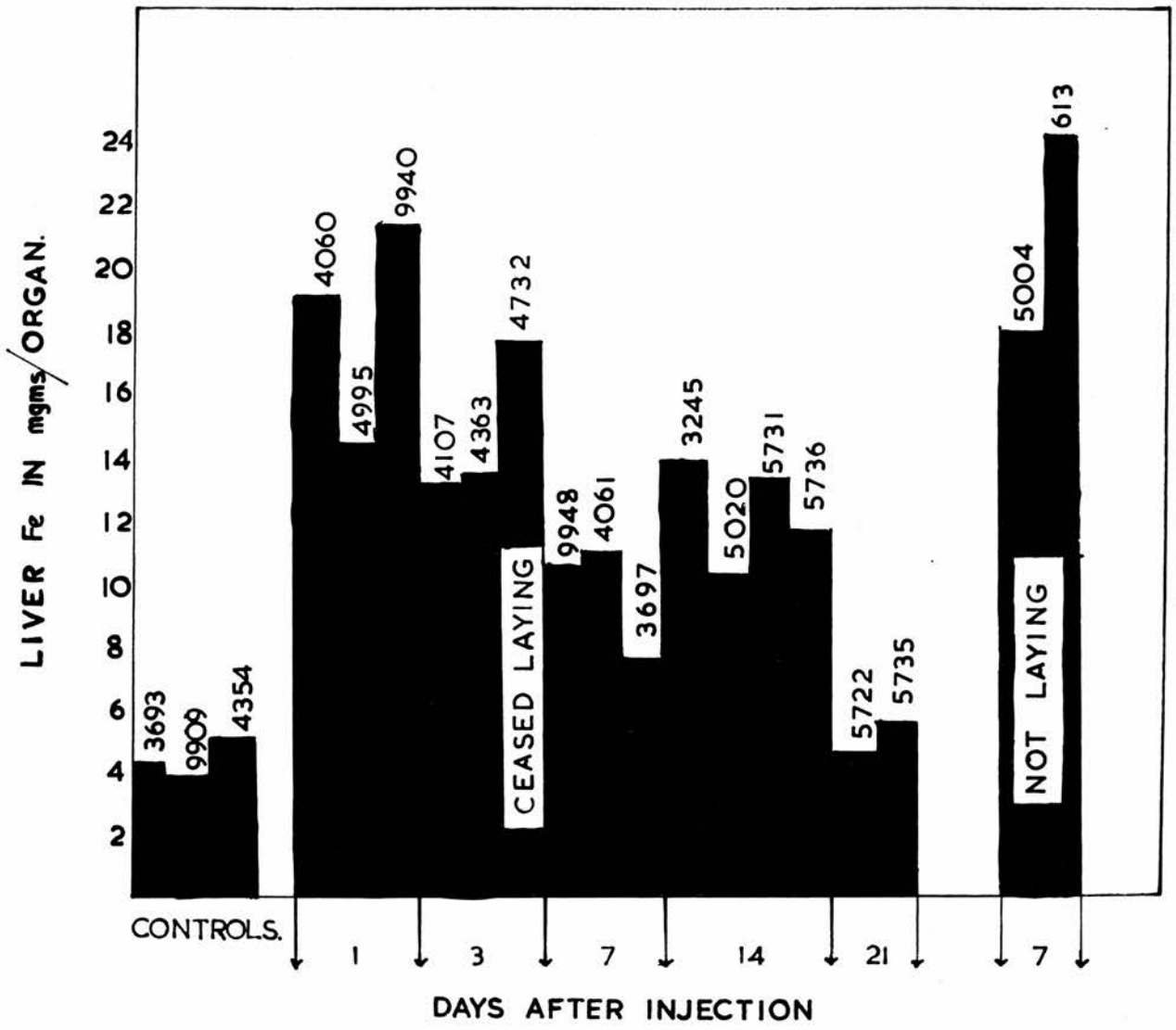
Control values for non haem iron averaged 4.2 mg per liver. Those birds killed after one day gave mean values of 18.4 mg; after three days, average values of 14.5 mg per organ were obtained. Twenty-one days after injection, the livers gave a mean value of 4.5 mg of non haem iron per organ (figs IV and V). With the two non-laying birds killed after seven days this fall was not apparent, nor in one bird which ceased laying. In a number of cases kidney and spleen non haem iron was also determined. The values found in laying birds were within the normal range in all cases.

Utilisation

The marked increase in storage iron following injection conforms with the picture in other animals where iron is essentially a 'one way element' (Finch et al 1950). In comparison with the controls some 14 mgms or seventy per cent of the saccharated iron was present in the liver and

FIG V

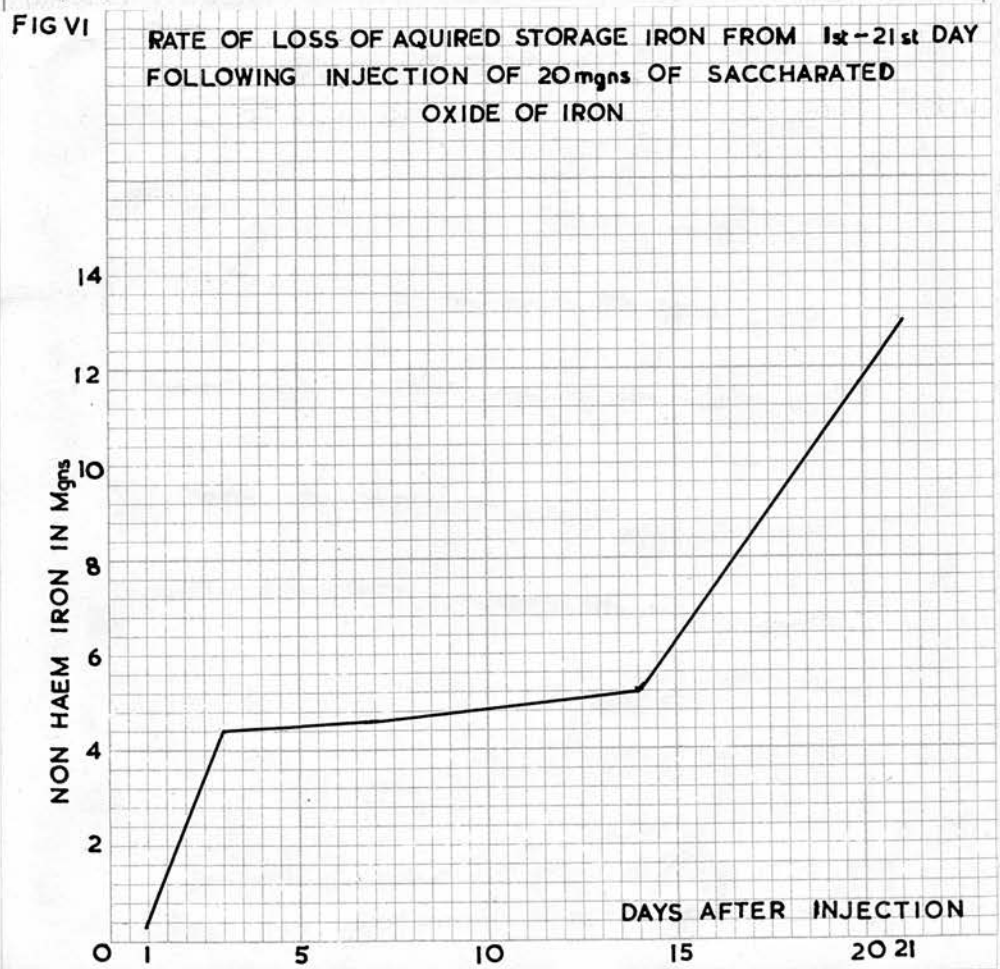
TOTAL LIVER NON HAEM IRON IN CONTROL AND INJECTED BIRDS



associated reticulo endothelial cells at the end of twenty-four hours. A further seven per cent was present in the eggs. Hawkins and Hahn (1944) found liver uptake of iron in the dog following injection of colloidal iron to be seventy-nine per cent. The two non-laying birds with no visible means of iron loss showed uptake of iron by the liver to be eighty five per cent. When the figures for kidney and spleen were included, ninety per cent of the injected iron could be accounted for.

When saccharated oxide of iron is injected in large amounts only traces are lost via the urine (Slack and Wilkinson 1949), and similar small losses of iron following injection occur via the bile (Hawkins and Hahn 1944), the greater part of the injected iron remaining in the storage organs.

FIG VI



The fate of the injected iron taken up by the body depots in the hen differs from that in the mammal. The curve (fig. VI) shows that the acquired iron was rapidly lost and suggests that liver iron was taken up by the developing yolks. If liver iron were going elsewhere, absorption of iron to meet the requirements of the developing ova would still be high and would result in an abnormally elevated plasma iron, composed of iron from absorption and from the liver. The results indicate that plasma iron returns to the normal level in a few days.

It is of interest that egg iron valves were within the normal range before liver non-haem iron had ceased falling and suggests that a large fraction of total egg iron, particularly towards the end of the experiment, was obtained from that injected.

Effect of Oestrogen on Iron Metabolism in Immature Pullets

Introduction

In mammals sex is known to influence the storage and utilisation of iron. Steenbock, Semb and van Donk (1936) and Wakem and Halem (1936) showed that the livers of female rats contained a greater concentration of iron than those of male litter mates, while female rats store some 12 per cent more iron than males having the same uptake per gram body weight (Rose and Hubbell, 1938). Ovariectomy resulted in decreased iron storage in the adult female, while injection of follicular hormone into immature females led to greater storage than occurred in control litter mates.

In poultry, Widdowson and McCance (1948) showed that pullets stored more iron in the liver than cockerels of the same age. Liver weight has been found to increase greatly in pullets following oestradiol injection (Chapman etal 1950). At the same time the total amount of 'inorganic' iron present rose, although the percentage figure fell.

The effect of sex on haemoglobin in birds has been mentioned previously, and the findings of Cook and Harmon (1933), and Tanaka and Rosenberg (1954), that good layers had lower haemoglobin values than poor producers, suggests a relationship with the oestrogenic hormone present. In the mammal a marked fall in haemoglobin occurs following oestrogen administration (Teague 1942).

Common, Rutledge and Bolton (1947, 1948) using the washing out technique reported an increase in plasma volume after feeding oestrogen. Sturkie (1951) using the dye

dilution method was unable to confirm this. Since these findings were relevant to the changes in haemoglobin, and further, since the washing out technique is known to be subject to great error, it was decided to re-investigate this problem. Preliminiary trials using laying birds conducted during the first part of the work had given widely varying and inconsistent results. One of the causes was found to be that dye was taken up by the eggs. Rodnan, Ebaugh and Fox (1957) using radio-active chromium found that it too was taken up by the eggs. By the use of immature pullets it was hoped to overcome this major source of error. Sturkie and Newman (1951) found no difference in the total plasma proteins of laying and non-laying hens, although oestrogen will greatly elevate total plasma proteins. This elevation is prevented by simultaneous injection of thyroxine. It was decided while plasma volume was being investigated to confirm this work, and study the simultaneous administration of anti-thyroid substances.

Fleischmann and Fried (1945) and Common et al (1947) found increased levels of serum calcium, phosphate, and protein following oestrogen administration, while Lorenz (1938) demonstrated marked increases in all lipoid fractions of the blood. Coincident with these changes, an increase occurs in the size and number of cells in the liver (Clavert 1944) as well as in total liver fat and protein (Common, Bolton and Rutlidge 1948). If thyroxine be administered simultaneously with oestrogen the oestrogen produced changes are inhibited (Common, et al 1947, 1948). Thiouracil if

given in large amount (2% of the diet) has a similar effect to thyroxine, but does not decrease liver weight or protein content (Common et al 1950). Certain sulphonamides behave similarly to thiouracil (McKenzie and McKenzie, 1943) and it was decided to use sulphamethazine in this work.

As these changes produced experimentally are associated with laying in the hen, it was deemed of interest to determine whether similar changes in iron metabolism could also be produced.

The experimental work was divided into three parts. Firstly, the effect of oestrogen on haemoglobin, plasma iron and liver iron in immature pullets was determined. Secondly, since throxine and goitrogenic agents have been shown to inhibit changes produced by oestrogen, it was decided to investigate their effect on any changes in iron metabolism produced by oestrogen, and concurrently to study alterations in plasma proteins.

Thirdly, blood volume was investigated before and after oestrogen therapy.

Experimental

Part I

Changes in Plasma Iron, Haemoglobin, and Liver Non Haem Iron Following Oestrogen Administration

General

In preliminary trials pullets between twelve and fourteen weeks of age were employed.

These birds were injected with 1 mg of oestradiol benzoate in arachis oil (one cc) three times weekly for three weeks, giving a total of 9 mg of oestradiol injected.

The results were of a uniform nature, but did not stimulate sufficiently the changes occurring naturally with the onset of laying. As the total amount of oestradiol injected was spread over twenty-one days and was considerably less than that given by Chapman et al (1950) who employed one large initial injection, the work was repeated using larger quantities of oestrogen.

Procedure

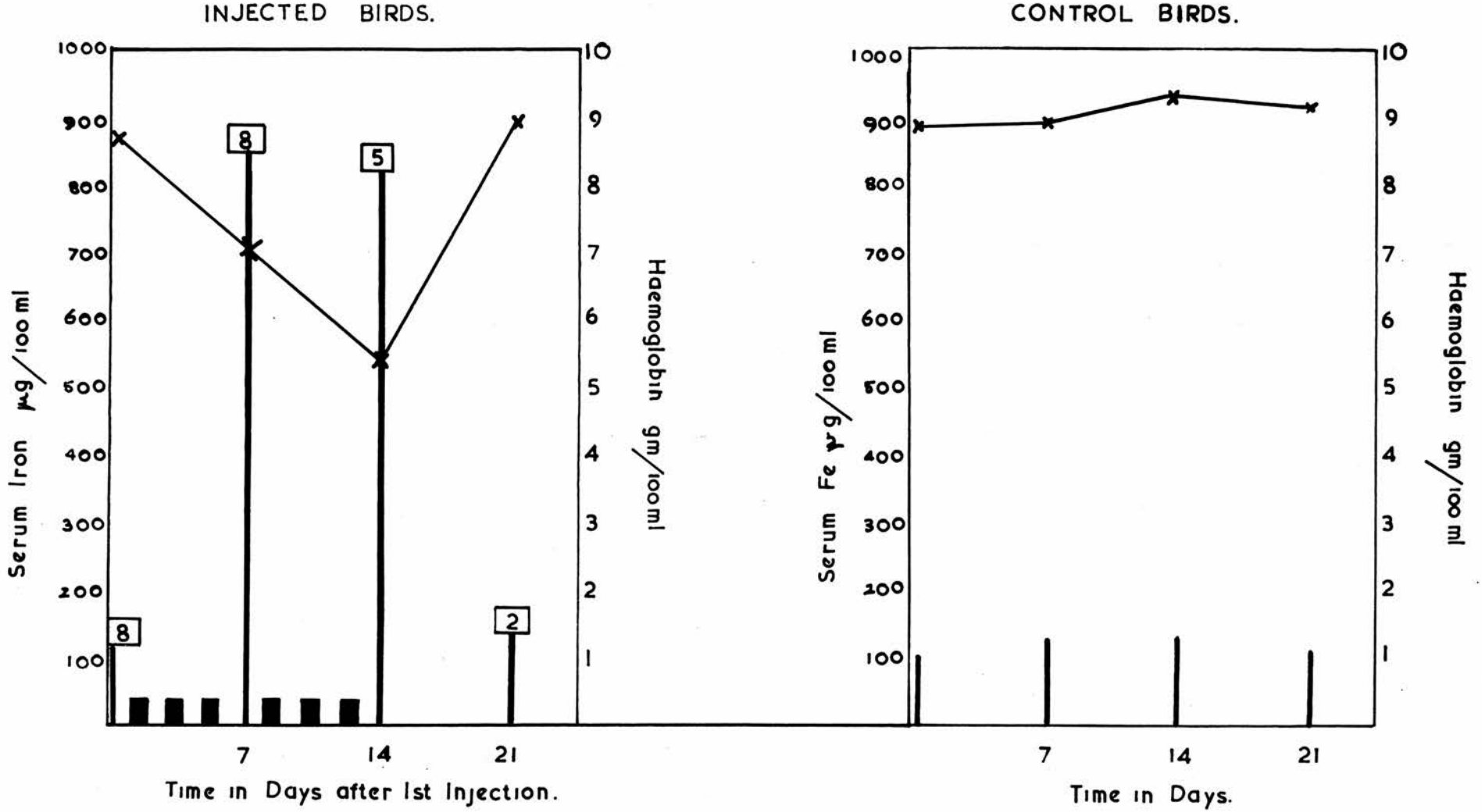
Twenty pullets of the same age as in the preliminary trials were selected and maintained in a deep little house where they had access 'ad lib' to food and water.

At the commencement of the experiment 5 ml of blood was taken from the alar vein of each bird to determine haemoglobin and plasma iron. At the same time, four were killed by separation of the cervical vertibrae, allowing free bleeding and determination of liver non haem iron in these birds was undertaken to establish control valves.

Of the remaining sixteen, eight were injected three times weekly with 3 mg of oestradiol benzoate in olive oil (1 ml), for two weeks, giving a total dosage of 18 mg of oestrogen. The remaining eight served as controls and were injected at the same time as the test group with pure sterile olive oil. After one week, 5 ml of blood was again taken from each bird for analysis, and three control and three injected birds killed to determine liver non haem iron.

FIG VII

MEAN SERUM IRON and HAEMOGLOBIN in CONTROL and INJECTED BIRDS.



Seven days later, after withdrawing 5 ml of blood from each of the remaining birds for analysis, three further control and three injected birds were killed. No further injections were given and the remaining four birds were sacrificed twenty-one days after the commencement of the experiment, i.e. seven days subsequent to the last injection.

Analytical Methods

Plasma iron, haemoglobin and liver non haem iron were determined as in the previous work.

Results

Plasma Iron

Mean control plasma iron levels throughout the experiment were consistent with results reported previously.

Injection of 9 mg of oestradiol benzoate in seven days resulted in a rise in serum iron to eight times the pre-injection level. Injection of a further 9 mg of oestradiol caused no further rise and within a week of discontinuing the injections values were back to the initial level (fig. VII).

Haemoglobin

A marked fall in haemoglobin was apparent after seven days and this fall continued up to when the level of oestradiol injected had amounted to 18 mg. One bird, whose initial haemoglobin had been 9.57 g/100 ml had a fall to 4.2 g/100 ml. Within a week of ceasing to inject oestrogen, haemoglobin values returned to normal levels (fig. VII).

Liver Iron

The preliminary experiments demonstrated a wide range of values for total liver iron. Similar findings were reported by Widdowson and McCance (1948) who found a range of 7 - 37.3 mg /100 ml of tissue.

The liver non haem values for the four birds killed as controls for both groups were lower, both in concentration and absolute amounts than those in the others of the control series, even if allowance is made for the 25 per cent. difference in liver weight between this and subsequent groups, or for any increase due to age, but is accountable for by the wide range of liver iron values found in the fowl (table XIII).

The amount of non haem iron in the livers of injected birds rose following administration of oestrogen. This was not seen when figures were expressed as concentration values due to the marked increase in liver weight which followed injection. This increase was more marked in those birds which had received the greater amount of oestradiol.

Oviduct

Marked development of the oviduct was present in all injected birds, but this had regressed somewhat in the week following injection. One control bird killed twenty-one days after commencement of the experiment presented slight evidence of development, probably associated with approaching maturity. In neither the injected nor control birds was any macroscopic sign of ovarian development noticed.

Table XIII

Mean Liver weights and Iron content of Control and Injected Birds

(Figures in parenthesis indicate the range of individual observations)

Injected Birds						Control Birds					
Amount Injected	Liver wt.	Liver non haem Fe per 100 mg tissue	Liver non haem Fe per Liver	Development of Oviduct	No. killed	Liver wt.	Liver non haem Fe per 100 mg tissue	Liver non haem Fe per Liver	Development of Oviduct	No. killed
Nil	24.8 gm (22-27)	6.5 (4.0-8.2)	1.65 (1.0-2.3)	Nil	4	24.8 g	6.5	1.65	Nil	4
9 mg	45.1 gm* (40-51)	10.3 (9.1-12.8)	4.7 (4.5-5.2)	well developed	3	28.1 g (23-30)	11.9 (7.3-15.2)	3.3 (2.1-4.1)	Nil	3
18 mg	47.9 gm* (45-53)	11.7 (8.0-17.0)	5.1 (3.9-6.4)	well developed	3	30.0 g (26-37)	13.6 (9.3-16.0)	4.1 (2.4-5.9)	Nil	3
18 mg	38.3 gm* (37.0-39.0)	8.3 (7.4-9.3)	3.2 (2.9-3.5)	enlarged	2	29.5 gm (24.0-33.0)	10.7 (10.3-11.2)	3.1 (2.8-3.5)	Nil to slightly + ve	2
7 days previously										

*Fatty infiltration

Part II

Changes in Plasma Iron, Haemoglobin and Plasma Proteins, in Immature Pullets resulting from Simultaneous Administration of (a) Oestrogen and Thyroxine, (b) Oestrogen and Sulphamethazine

Procedure and General

Twelve leghorn pullets of the same age as in the previous experiment, but from a different flock, were maintained on deep litter as before. On this occasion central heating was not present, but it was not felt that any changes in temperature encountered would greatly affect thyroid metabolism.

All twelve received 18 mg of oestradiol benzoate over fourteen days as previously. In addition four of the birds received 1 mg of thyroxine daily intramuscularly for the fourteen days.

This daily level of thyroxine was the same as that given by Fleischmann and Fried (1945) and Common et al (1948), but more than would have been obtained by the birds of Kumaron and Turner (1949b) who received thyroprotein in the diet. A further four of the birds were fed sulphamethazine in the diet at the levels employed by Asplin et al (1947) and Turner and Kumaron (1949), i.e. 0.2% or 9 mg per 10 lbs feed. The remaining four birds were used as controls on the action of oestradiol benzoate.

Blood was withdrawn before the commencement of treatment and then at weekly intervals during the course of the experiment. To ensure that the sulphamethazine was being received effectively clotting time was measured for each of the four birds concerned when blood samples were obtained.

In all cases this was prolonged, an agreement with the findings as Asplin et al (1948). In one bird the clotting time was actually lengthened to ninety minutes.

No difference in weight or comb size were observed between the three groups at the termination of the experiment. The birds receiving thyroxine appeared to shed more feathers, but this could in no way be compared to moulting.

Methods of Analysis

Plasma iron and haemoglobin were determined as before. Total proteins were determined by biuret. Protein separation was by paper electrophoresis, employing the method of Jenks, Jetton and Durrum (1955). No attempt was made to stain for, or estimate the HLV fraction of McKinley et al (1953) although sera from all birds except those receiving thyroxine showed a marked yellow opacity.

Plasma calcium was determined by the method of Clarke and Collip (1925).

Results

Plasma Iron and Haemoglobin

The birds receiving oestradiol alone showed a similar rise in plasma iron (Table XIV) to that reported previously, with a return to normal values seven days after cessation of injection; haemoglobin over the same period declining and then rising when administration of the hormone ceased. Injection of thyroxine resulted in inhibition of oestrogen response both on plasma iron and haemoglobin. As a check on these findings plasma calcium which had been determined

at the start of the experiment, was redetermined after 14 days. The figures which are included for comparison agree with those of Common et al (1948). The birds receiving sulphamethazine showed marked augmentation of oestrogenic effect on plasma iron and haemoglobin as well as on blood calcium. If this sulphonamide is goitrogenic the findings are in contradiction to those of Common et al (1950).

Total Plasma Proteins

As can be seen from Table XV oestrogen caused an increase in total plasma proteins which was inhibited by injection of thyroxine, confirming the results of Sturkie (1951).

The further claim by Sturkie that administration of thyroxine actually caused a lowering of total plasma protein could not be investigated as rapidly growing birds were used.

The effect of sulphamethazine would again appear to be one of augmentation of the activity of oestrogen

Table XIV

Plasma Iron and Haemoglobin Levels

All birds were injected with 3 mg Oestradiol Benzoate thrice weekly for 14 days; Plasma Iron in ug/100 ml; Haemoglobin in g/100 ml; Ca in mg/100 ml

Bird No.		Pre-injection Values	Days after start of injection		7 days after last injection
			7	14	
		Oestradiol Benzoate only			
1	Plasma Fe	170	500	925	180
	Hb	9.9	8.9	7.5	9.7
	Ca	18		67	
2	Plasma Fe	155	600	1160	137
	Hb	10.1	8.9	7.1	9.6
	Ca	19.2		71	
3	Plasma Fe	195	560	1120	130
	Hb	9.6	8.4	6.8	9.9
	Ca	19.6		66	
4	Plasma Fe	135	550	1090	105
	Hb	10.2	8.2	6.7	9.8
	Ca	17.4		72	
		Oestradiol Benzoate + 1 mg Thyroxine for 14 days			
5	Plasma Fe	160	275	250	90
	Hb	10.3	9.2	8.7	10.7
	Ca	18.6		16.2	
6	Plasma Fe	140	140	160	130
	Hb	10.9	10.1	10.2	10.3
	Ca	17		16	
7	Plasma Fe	150	175	140	185
	Hb	9.4	9.8	9	9.2
	Ca	17		-	
8	Plasma Fe	190	160	180	145
	Hb	9.7	8.7	10.2	9.3
	Ca	14.4		14.2	

Table XIV continued

Bird No.		Pre-injection Values	Days after start of injection		7 days after last injection
			7	14	
Oestradiol Benzoate + 0.2 Sulphamethazine in feed for 14 days					
9	Plasma Fe	130	660	1070	160
	Hb	10.3	8.7	6.7	9.7
	Ca	18.2		74	
10	Plasma Fe	115	870	1350	135
	Hb	10.4	8.3	6.2	10.1
	Ca	15.8		82	
11	Plasma Fe	150	910	1285	125
	Hb	9.2	7.7	5.3	9.6
	Ca	14		73	
12	Plasma Fe	220	950	1390	168
	Hb	10.1	8.0	6.2	9.5
	Ca	14.4		79	

Table XV

Changes in Total Plasma Protein of 12 - 14 week old pullets following administration of (a) Oestrogen; (b) Oestrogen + Thyroxine; (c) Oestrogen + Sulphamethazine

Group	Before Treatment	Days after start of Treatment		Days after cessation of treatment 7
		7	14	
A Oestrogen 3 x 3 mg/ 7 days	4.25 g/ 100 ml	6.65 g/ 100 ml	6.5 g/ 100 ml	4.3 g/100 ml
B Oestrogen 3 x 3 mg per 7 days + 1 mg Thyroxine daily	4.5 g/ 100 ml	4.3 g/ 100 ml	4.9 g/ 100 ml	4.7 g/ 100 ml
C Oestrogen 3 x 3 mg per 7 days + 0.2 Sulphamethazine in feed	4.45 g/ 100 ml	6.5 g/ 100 ml	7.5 g/ 100 ml	4.1 g/ 100 ml

Fractionation of the Plasma Proteins

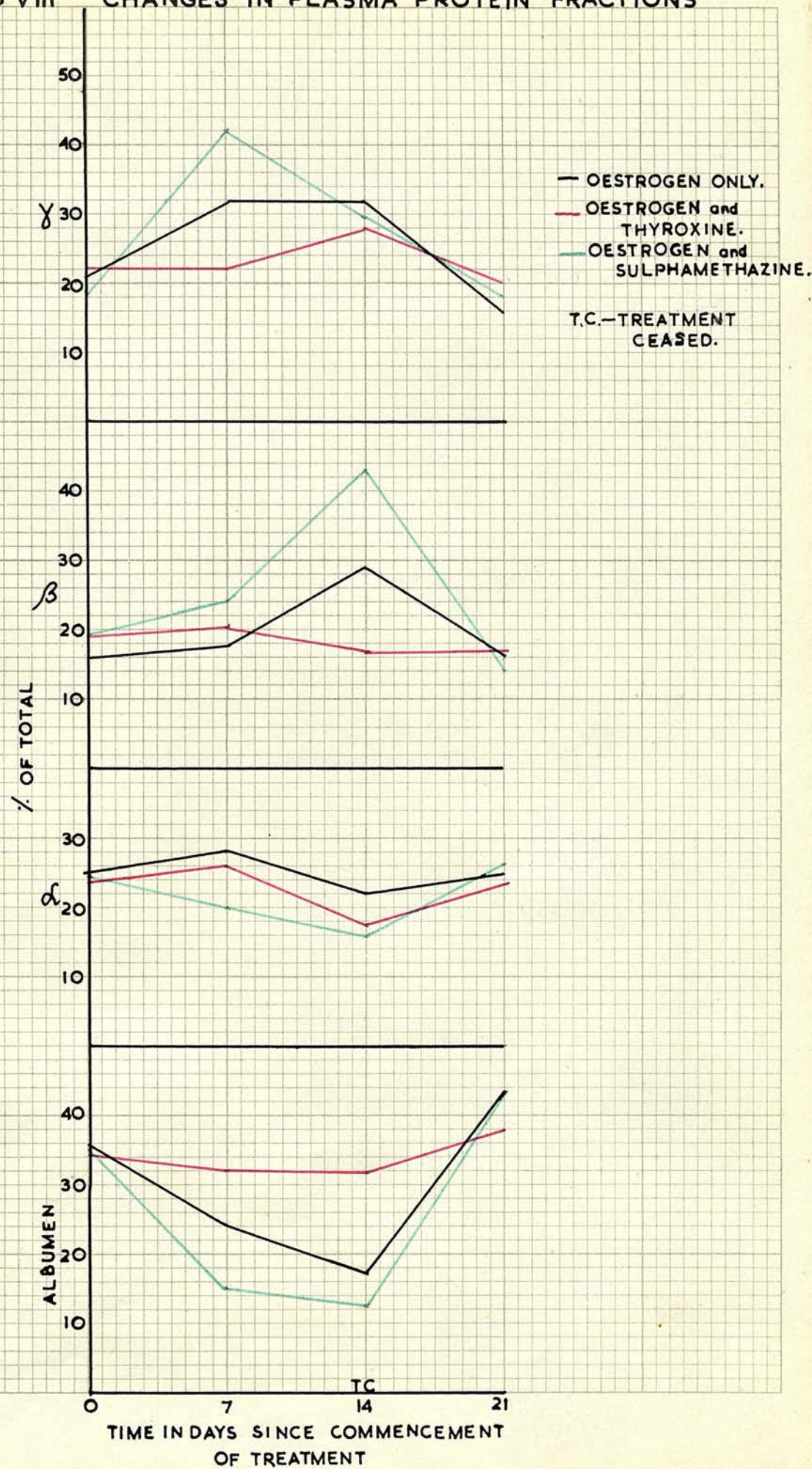
On electrophoresis, the plasma protein fractions defined by Common et al (1953) for immature pullets were obtained, quantitative values being within the range given by Campbell (1957). The addition of oestrogen produced the typical picture found in laying birds (fig. VIII).

With those birds given sulphamethazine the rise in globulin and fall in albumen was even more marked, and the three globulin components could not be readily identified. Further a very dark band was apparent in all tracings at the line of application of the serum, and presumably represents the pseudo lipovitellin (PLV) fraction of McKinley et al (1953).

Sturkie (1951) using the salting out technique, claimed that the albumen globulin ratio was not altered following oestrogen administration, although he obtained a 60 per cent rise in total plasma protein. It may be in the present experiment that the increase in β and γ globulins is in part due to the spreading of the PLV fraction, but against this suggestion is the absence of increase in the α globulin component despite the presence of an α globulin lipoid band stainable by osmic acid (McKinley et al 1953).

The thyroxine fed birds showed a slight increase in γ globulin with a corresponding fall in the α and β components during the last few days of treatment. These changes may have progressed had thyroxine continued to be administered.

FIG VIII CHANGES IN PLASMA PROTEIN FRACTIONS



Part III

Effect of Oestrogen on Blood Volume of Immature Pullets

General

For this experiment twelve pullets were employed. The birds averaged sixteen weeks of age and were housed and fed as in the previous work. All birds were weighed individually at the commencement and end of the experiment.

Experimental

The birds were divided into two groups of six. One group received 3 mg of oestradiol benzoate thrice weekly for 14 days, the other group acted as controls.

To ensure that the administered oestrogen was effective, haemoglobin was determined before and after oestrogen administration. In all injected birds the lowering of haemoglobin was similar to that found in parts I and II.

Blood volume was determined using T 1824. In earlier work various concentrations of the dye had been employed using laying birds and allowing an interval of ten minutes after injection before withdrawal of the dyed sample. Results were inconsistent, one of the causes being attributed to the fact that the dye was absorbed by the yolks. A further source of error was the possibility that the time between injection of the dye and withdrawal of sample for estimation was too long. Newell and Shaffner (1950) employing an interval of ten minutes obtained consistently higher results than Pino et al (1951) who allowed three minutes for mixing.

Before the start of the present work preliminary trials were again conducted on laying birds, 2.5 mg of dye being

injected and a mixing time between 3 and 5 minutes allowed. From results obtained it was decided to inject 2.5 mg of dye and allow a mixing time of 3 minutes.

Method

5 ml of blood was withdrawn from an alar vein, and without removal of the needle 2.5 mg of T 1824 were injected. After allowing 3 minutes for mixing, 4 ml of blood was taken from the opposite vein.

Blood from the undyed sample was taken for haematocrit and red cell smears to check axis diameters. Both samples were then centrifuged and 2 ml of the supernatent taken for determination of dye dilution.

A standard was set up for each bird and consisted of .08 mg of T 1824 contained in a volume of 0.2 ml added to 1.8 ml of undyed serum. All readings were taken at 620 mμ in a Unicam S.B. 350 spectrophotometer. Zero setting was determined for each bird individually using undyed plasma.

Total Blood Volume was determined using the formula:

$$\text{Blood Volume} = \frac{\text{Plasma Volume}}{1 - \text{Haematocrit}}$$

Haematocrit was determined by spinning at 3,500 r.p.m. for 45 minutes. Cell diameters were measured using a micrometer eye piece.

Results

Total blood volume of control and injected birds is given in Table A. Values before oestrogen therapy and for the control birds are in agreement with those of Pino et al (1951) for Leghorn birds. As the birds were increasing in

weight over the experimental period and as the numbers were too small to establish a weight/blood volume relationship, the increase in blood volume was expressed as a percentage of the initial volume.

While growth undoubtedly affects blood volume, those birds receiving oestrogen showed significant increase over the controls. One bird (304) although apparently healthy had lost weight. This was reflected in the change in blood volume.

Red cell diameters were in all birds within the normal range (Sturkie 1954) being between 11.8 - 13.0 microns for the long axis and 6 - 7 microns for the short.

Table XVI

Changes in blood volume following oestrogen injection

Control Birds

Bird No.	Weight at commencement Kgs	Blood Volume ccs	Blood Volume ccs/Kg	Haematocrit	Haematocrit ccs.rbc/Kg	Weight at termination	Blood Volume ccs	Blood Volume ccs/Kg	Haematocrit	Haematocrit ccs.rbc/Kgs	% increase in Blood Volume
307	1.410	78	56 55	34	× 19.4 18.7	1.730	108	× 60 63	32	20.5 20.1	38.5
308	1.190	62	53 52	33	17.4 17.1	...	...	...	sample haemolysed	...	... ...
309	1.250	76	61	32	19.8 19.5	1.430	102	70 71	34	× 22.5 24.1	34
310	1.135	82	72	38	27.5 27.3	1.310	110	84	35	29 29.4	34
311	1.160	81	72 70	33.5	23.5 23.4	1.310	122	92	37	35 36.0	50.5
312	1.010	58	58	37	21.5 21.4	1.050	90	× 90 86	33	× 29.5 28.3	55

Treated Birds	Before Injection					After 18 mg Oestradiol Benzoate					
Bird No.	Weight at commencement Kgs	Blood Volume ccs	Blood Volume ccs/Kg	Haematocrit	Haematocrit ccs.rbc/Kg	Weight at termination	Blood Volume ccs	Blood Volume ccs/Kg	Haematocrit	Haematocrit ccs.rbc/Kgs	% increase in Blood Volume
301	1.160	77	66	35	23.0 23.1	1.340	127	95	31	29 29.5	65.5
302	1.025	65.5	63 64	37	23.5 23.6	1.190	135	× 88 114	26	29.3 29.6	106
303	1.145	68	× 67 59	33	× 18.0 19.4	1.285	173	× 75 135	25	34 38.8	154
304	1.160	80	69	35	24.0 24.1	0.995	107	× 100 107	23	24.5 24.6	34
305	1.165	71	61	34	20.5 20.7	1.360	147	× 100 108	28	30.5 30.2	108
306	1.270	74	59 58	335	19.8 19.4	1.490	147	100 99	28	28 27.7	98.5

Discussion

Blood Volume

The increase in blood volume following oestrogen therapy is in agreement with the findings of Common et al (1947) who used a washing out technique.

As no change in red cell size was observed the fall in haemoglobin per 100 ml blood in oestrogenised birds could be due to either a decrease in the total number of circulating red cells, or an increase in plasma volume. In the former case, if plasma volume remained constant total blood volume would have fallen. As the opposite was observed the decreased haemoglobin in oestrogenised birds must be the result of increased plasma volume. This is supported by the fact that in the rapidly growing pullets investigated the red cell count/Kg increased in the treated as well as in the control birds.

Rodnan et al (1957) using red cells tagged with radioactive chromium, to determine red cell volume in birds, claimed that those with lowered haemoglobin had a lower red cell volume and suggested that as hens had half the red cell volume of roosters, but were not iron deficient, erythropoesis must be slower. Their method of calculation, however, is based on the assumption that plasma volume in all cases is equal. Newell and Shaffner (1950) have demonstrated that in hens the relationship between body weight and blood volume is not linear.

Haemoglobin

The haemoglobin values found in this study confirm that a fall in haemoglobin occurs in laying birds as reported by Cook and Harmon (1933), Maughan (1935), and Tanaka and Rosenberg (1954).

The fall in haemoglobin was less than reported by Cook and Harmon, but this may be explained by the wide range of values reported by these workers, one of their birds having values ranging from 5 - 19 gm Hb/100 ml in one season. The contrary findings of Dukes and Schwarte (1931), Winter (1936) and Schultze and Elvehjem (1936) may be explained by the work of Tanaka and Rosenberg (1954), who found that the mean differences in haemoglobin between laying and non-laying birds in a flock was not significant, due to the wide range of haemoglobins encountered, but a study of individual birds showed a significant fall with laying.

As the decrease in haemoglobin would only provide some 3 - 5 gm of iron for the eggs and as the laying level of haemoglobin remains more or less constant despite continued iron loss, it is unlikely that iron deficiency is primarily responsible. The finding that liver iron does not reduce to zero supports this conclusion and, further, a marked rise in iron stores following injection of iron caused no rise in haemoglobin levels.

The marked increase in plasma volume following oestrogen administration, supports the contention that the fall in haemoglobin is essentially due to blood dilution and confirms the findings of Dom and Taber (1946) that poulards treated

with oestrogen had a decreased red cell count. Similar effects of oestrogen on haemoglobin have been reported in rats (Teague 1942) and dogs (Tislowitz 1939). As no complementary increase in size of the red cell occurs, this lowering of haemoglobin must result in less oxygen reaching the tissues in unit time, unless there is a compensatory increase in circulation rate, a problem which has not yet been investigated.

Plasma Iron and Egg Iron

In the mammal an increase in plasma iron may represent increased red cell destruction or conversely decreased bone marrow activity (Laurell 1947). Increased iron requirement or continued loss results in lowered serum iron as reserves become depleted. In the bird, despite continued loss via the egg, a decrease in plasma iron is not seen, and suggests, particularly as liver iron falls although it does not reach zero, that plasma iron levels do not reflect the depth of iron reserves.

Cartwright et al (1950) explained the high serum iron seen in some pathological conditions on the assumption that the serum level indicates iron available for haemoglobin synthesis. In the hen the high value obtained is more representative of requirements of the developing ova than of the bone marrow.

A study of values of egg iron and plasma iron during the laying period suggested a relationship between plasma iron and the amount excreted in the egg, and further comparison gives a correlation coefficient of 0.717 ($P<0.001$) for egg iron and

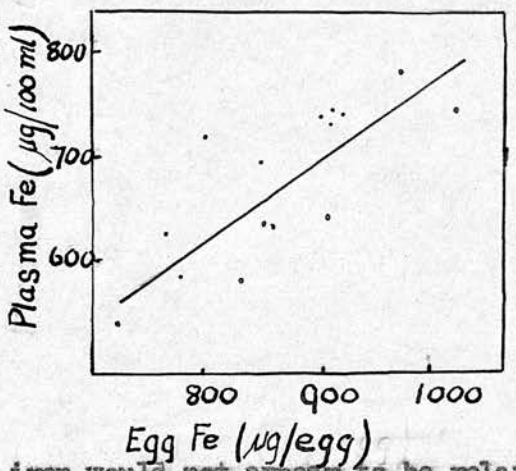
plasma iron and is consistent with the view that egg iron is derived from plasma iron (fig. IX). Further confirmation is afforded by the finding that the elevation of plasma iron following injection of iron, resulted in an increased amount in the egg. If this increase in egg iron had been due entirely to the saccharated iron present, saturation of the iron binding capacity of the plasma followed by deposition of iron in the tissues could have been responsible, but an increase in both fractions was present as long as plasma iron remained elevated. In the case of ferrous ascorbate, removal by the storage organs must have been rapid.

The relation between egg iron and plasma iron

Fig. IX.

The points were obtained by plotting the mean egg iron over four months against the mean plasma iron (determined every three weeks) during the same period. Fifteen birds were used.

The straight line was calculated from the correlation coefficient $r = 0.717$ (P 0.001).



The elevated plasma iron would not appear to be related to loss via the egg, since it can be reproduced in non-laying oestrogenised pullets and the stimulating factor is probably hormonal. Further, a relationship between the level of plasma iron attained and the strength of the stimulus was apparent in preliminary trials with oestradiol. Where 3 mg of the compound was injected weekly over three weeks, no rise in

plasma iron over 300 ug/100 ml was observed. Trebling the amount injected, raised plasma iron to over 900 ug/100 ml. As storage iron also increased it is apparent that the rise in plasma iron is derived from increased oral absorption.

As changes in other plasma constituents are seen at laying (Common et al 1947) it would appear that the elevation in plasma iron at ovulation is part of the "maturation syndrome", or secondary sexual changes which occur in blood constituents at laying. These changes as far as iron metabolism is concerned do not appear to be initiated or terminated by the factors which govern iron metabolism in the mammal. However, once established they then appear to conform to an accepted pattern which resembles that of the mammal, plasma iron being representative of internal iron activity and absorption being regulated by the need of the body for iron.

While the initiation and termination of these maturation changes may be related to the amount of circulating oestrogen, the effect of thyroid and goitrogenic agents suggests a more primary factor, possibly the pituitary, and the work of Crafts and Walker (1947), who showed that serum iron was decreased and storage iron increased in hypophysectomised rats, strengthens this suggestion.

Absorption

It has been suggested(Granick 1949),that the synthesis and breakdown of apoferritin is a continuous process in which breakdown is prevented only by the presence of iron;

or that the synthesis of apoferritin is stimulated by the presence of iron with breakdown occurring upon removal of iron (Michaelis 1947). This synthesis occurs until the cell is saturated with iron as the protein complex ferritin. In normal animals little ferritin can be demonstrated (Gabrio 1950), but on feeding iron, even though no iron deficiency exists, the content of ferritin is greatly increased. The amount of ferritin would therefore appear in the normal animal to be related to the available iron present up to the limit when the cell is saturated. If this holds in the laying hen, two possibilities exist, (a) the amount of 'available iron' must increase considerably at ovulation since the rate of absorption is controlled by the content of ferritin in the intestinal mucosa (Granick 1949). This increased availability occurs on a cereal diet rich in phytate, which would be expected to exert an adverse effect (Widdowson and McCance 1942). It may be calculated from iron intake and egg iron that the laying bird must have absorbed some 15-20 per cent of the dietary iron. The presence of any component with a high reducing effect would conversely augment absorption (Moore and Dubach 1951). (b) Alternatively, there may be the same amount of 'available iron' present, but release of iron into the blood stream from the intestinal cell may be more rapid. This, according to Granick, is dependent upon the oxygen level of the blood which is determined by the haemoglobin level. In oestrogenised pullets the fall in haemoglobin coincident with increased plasma iron would

appear to support this hypothesis, but in laying birds absorption may cease when reserves are high, while haemoglobin still remains at a lowered level. Dubach et al (1948) have shown that increased absorption is not related to plasma iron levels. Laurell (1947) has suggested that the degree of saturation of the iron binding component of the plasma may affect absorption. This aspect has not yet been investigated in the fowl, and it would appear that in the laying hen absorption is dependent on the need of the body for iron and is not related to plasma iron levels per se, nor the oxygen level of the blood.

Storage Iron (Liver Iron)

Breneman (1942) reported marked fatty infiltration in chicken livers following oestrogen administration. This was confirmed by Stamler et al (1950) who reported that the increase was chiefly due to fatty acids and cholesterol. Similar infiltration and enlargement was noticed in the present work both at onset of laying and following oestrogen injection.

The figures for non haem iron 1.9 mg - 9.5 mg per liver are in agreement with the wide range reported by Widdowson and McCance (1948) and Common et al (1950), the higher figures being found among birds which were not laying.

The high daily loss of iron in laying birds suggests eventual deficiency of reserve iron, which in time would permit increased uptake of iron from the serum, and a study of this point from the rate at which plasma iron falls after intravenous injection of iron is of interest.

The rapidity of elimination of iron from the blood stream is dependent on: (a) the iron binding capacity of the serum; (b) the activity of the bone marrow; (c) uptake of iron by the ova; and (d) levels of storage iron. In man an induced rise of plasma iron to saturation limit will revert to normal in 24 hours and no increase in the iron binding capacity of the serum occurs (Laurell 1947), and it is improbable that the slow fall in serum iron seen in the hen is due to alteration in the iron binding capacity of the serum. As no increased erythropoesis was observed, the elevated plasma iron after three days, despite increased loss via the eggs, is suggestive of slow uptake by the storage organs, and supports the conclusion that in laying birds no state of iron deficiency exists. Evidence for the alternative hypothesis, that regulation of absorption is poor and intake still continued despite loaded iron stores, is weakened by the fact the non laying birds with no ready source of iron loss, and much reduced absorption, still had an elevated plasma iron when killed seven days later. Further, one bird which ceased laying had a rapid fall in serum iron. The first part of the work indicated that birds on cessation of laying increased their storage iron, i.e. acted as iron deficient.

Studies using Fe^{55} & ^{59} in normal species (Finch et al 1949) have shown that about fifty per cent of the injected iron which has been located in the liver is used for haemoglobin production in 2 - 3 days, signifying that the intravenous iron did not mix completely with iron stores, but entered the 'labile pool'. This did not hold when

massive doses were administered. Whether a 'labile pool' exists in the laying bird which has only 3 - 4 mg total reserve iron is questionable, since daily turnover of iron is about half this amount as the life of the avian RBC is only twenty-eight days. Thus in a bird with some 8.3 gms of haemoglobin, daily haemoglobin breakdown would be 8/28 or 0.3 gm Hb equivalent to one milligram of iron. A similar amount is lost in the egg daily, and it is therefore more probable that complete mixing in storage occurs. In the non-laying bird with higher amounts of depot iron, incomplete mixing of reserve iron and that eliminated from the serum may occur, since injected iron was not used to increase haemoglobin. Isotope studies would enable this point to be settled.

The fall in liver non haem iron at the commencement of laying is of interest, but can in no way account for the iron lost in the eggs (although this is less than the average in the first few weeks of laying) and suggests that the mechanism controlling absorption may develop gradually, depot iron being utilised to correct deficiencies. The continued efficiency of this mechanism, when established, is displayed by the negligible fall in storage iron during laying. It is unlikely that the hormonal stimulus for increased absorption is affected by the level of depot iron, since the oestrogenised pullets had no known source of iron loss. It is more probable that the hormone is more concerned with the level of circulating iron, and provided this is adequate for laying requirements storage levels and absorption are related by a pattern similar to that found in the mammal.

Conclusion

This work has shown that a heavy loss of iron via the egg occurs in the hen while laying. To adapt the body to this constant demand upon reserves and maintain the "milieu intérieur" of Claude Bernard, the hen has acquired the faculty of increasing its absorption of iron from the gut to prevent depletion from the storage organs reducing body reserves to zero.

The physiological mechanism initiating this increased absorption by the intestinal mucosa appears to be hormonal; the faculty being lost on cessation of ovulation. The level of iron in the storage organs appears to regulate the actual amount of iron absorbed. Injected iron is taken up by the depots, as in the mammal, but is not retained, being rapidly transferred to the eggs. During this phase plasma iron values indicate that absorption is diminished until storage iron levels have returned to their pre-injection level.

The lowered haemoglobin encountered in laying birds is associated with plasma dilution and is not the result of depletion of iron reserves by laying.

Results similar to the changes which occur with laying can be produced by injection of oestrogen. These changes are reversible, withdrawal of the stimulus, resulting in a return to pre-injection levels.

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